Identification of *Neisseria lactamica* Outer Membrane Proteins Protective against Meningococcal Disease

Thesis

Identification of *Neisseria lactamica* Outer Membrane Proteins Protective against Meningococcal Disease

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Abstract

A number of approaches have been taken towards the development of a vaccine protective against serogroup B meningococcal disease but, as yet, none have been successful. The commensal bacterium, *Neisseria lactamica*, shares many surface structures with *N. meningitidis* and *N. lactamica* may therefore provide an alternative approach to vaccinating against serogroup B disease. Immunological evidence suggests that carriage of *N. lactamica* is involved in natural protection against disease caused by *N. meningitidis*.

This study presents the important observation that *N. lactamica* vaccines protect mice against meningococcal challenge. To identify the components responsible for protection, the outer membrane proteins of *N. lactamica*, extracted from whole cells, were separated by preparative electrophoresis and pooled into low (<43 kDa), medium (43-65 kDa) and high (>67 kDa) molecular weight protein groups. The low molecular weight group provided the best protection of these groups, and further separation of this group indicated that proteins of 25-43 kDa provided the observed protection. Serum raised against *N. lactamica* proteins was cross-reactive with meningococci of different serogroups, serotypes and serosubtypes. *N. lactamica* antisera raised in mice were not bactericidal and although sera raised in rabbits showed some bactericidal activity, titres did not correlate with protection.

Meningococcal proteins cross-reactive with *N. lactamica* antisera were identified using surface enhance laser-desorption ionisation time-of-flight mass spectroscopy. The cross-reactive proteins had molecular masses of approximately 11.2 kDa, 13.7 kDa, 26.8 kDa, 17.4 kDa, 28.1 kDa, 33.1 kDa, 53.2 kDa and 66.6 kDa. Several meningococcal proteins of unknown function and others that have previously been considered as vaccine antigens (PorB and TbpB) were putatively identified. Proteins with epitopes homologous to these proteins are likely to be present in *N. lactamica* and may be involved in protection against meningococcal challenge. The identity of the 66.6

kDa protein as TbpB was confirmed by comparing the cross-reactivity of *N. meningitidis* OMPs from wild-type and TbpB knockout strains with *N. lactamica* antisera.

Using a *N. lactamica* genomic expression library, the DNA sequences of recombinant *N. lactamica* proteins cross-reactive with *N. lactamica* antiserum were obtained. Meningococcal proteins with homology to the *N. lactamica* sequences were identified by comparison with the complete genome sequences of *N. meningitidis* serogroups A and B. Fifteen cross-reactive sequences coded, either partially or completely, for 23 different proteins.

This study demonstrates that *N. lactamica* provides an effective vaccine in mice against lethal meningococcal challenge and that *N. lactamica* may provide an alternative approach to vaccination against serogroup B meningococcal disease. Putative identifications of the proteins involved in this protection have been made.

Declaration

I declare that the research presented in this thesis is all my own work except for the following: Animal licensed procedures were carried out with my help by Dr Karen Reddin at CAMR. This work has not been submitted elsewhere for a research degree.

KJ Setchfield

Kerry Setchfield

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Dedication

To Rich, for your support, understanding, encouragement, help and for believing in me.

To my family for all of your support, help and encouragement.

Publications

Full Refereed Publications

Neisseria lactamica Protects Mice Against Experimental Meningococcal Infection. Oliver K.J., Reddin K.M., Bracegirdle P., Hudson M.J., Borrow R., Feavers I.M., Robinson A., Cartwright K., Gorringe A. Infect. Immun. 2002; **70**(7): 3621-3626

Poster Presentations

Use of SELDI to Identify Meningococcal Proteins that are Recognised by Sera Raised against *Neisseria lactamica*. K. Setchfield, B.J. Sheehan, K. Reddin, A.R. Gorringe, J.S.Kroll, P.R.Langford. Ciphergen Symosium, Belgium. 2002

Neisseria lactamica Proteins Protect Mice against Meningococcal. K. Oliver, P. Bracegirdle, K.M. Reddin, R. Borrow, A.J. Fox, I. Feavers, M.J. Hudson, A. Robinson, K.A.V. Cartwright, A.R. Gorringe. 12th IPNC, Galveston,USA. 2000

Neisseria lactamica Provides a Cross-Reactive Vaccine against Meningococcal Disease A.R. Gorringe, K. Oliver, P. Bracegirdle, K. Reddin, M. Hudson, K. Cartwright, I. Feavers, R. Borrow and A. Robinson. 12th IPNC, Galveston,USA. 2000

Neisseria lactamica as a Vaccine against Meningococcal Disease
K. Oliver, P. Bracegirdle, K. Reddin, A.R. Gorringe, R. Borrow, A.J. Fox, I. Feavers, M.J. Hudson, A. Robinson, K.A.V. Cartwright. PHLS Conference, Warwick. 2000

Cross-Reactivity of *Neisseria lactamica* with Meningococcal Antigens. P. Bracegirdle, K. Oliver, A.R. Gorringe, R. Borrow, A.J. Fox, I. Feavers, M.J. Hudson, A. Robinson, K.A.V. Cartwright. PHLS Conference, Warwick. 2000

Neisseria lactamica as a Vaccine against Meningococcal Disease. P. Bracegirdle, K. Oliver, K. Reddin, A.R. Gorringe, R. Borrow, A.J. Fox, I. Feavers, M.J. Hudson, A. Robinson, K.A.V. Cartwright. PHLS Conference, Warwick. 2000

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Submitted Abstracts

Identification of potentially protective *Neisseria lactamica* proteins using an *N. lactamica* genomic library. K.J Setchfield, I.D Beech, K.M Reddin, M.J Hudson, A.R Gorringe. 13th IPNC, Oslo, Norway. 2002

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Identification of *N. lactamica* with Potential for Inclusion in a Vaccine against Meningococcal Disease. I.D Beech, K.J Setchfield, K.M Reddin, C O'Dwyer, M.J Hudson, A.R Gorringe. Cold Spring Harbour Lab Meetings, USA. 2002

Patents

Immunogenic Commensal *Neisseria* Sequences. Inventors: Oliver K.J., Gorringe A., Hudson M., West D. UK Patent Application Number 0107219.8, 22 March 2001

Contents

List of Abbreviations

1D	One Dimensional
2D	Two Dimensional
A	Adenine
APS	Ammonium Persulphate Solution
b.p	Base Pairs
BB	Bactericidal Buffer
BCA	Bicinchonic Acid
BHI	Brain Heart Infusion Agar
°C	Degrees Centigrade
C	Cytosine
CAMR	Centre for Applied Microbiology and Research
Cap	Capsule
CFU	Colony Forming Units
cm	Centimetre
CO_2	Carbon Dioxide
CPS	Capsular Polysaccharide
CRM	Cross-Reactive Material of Modified Diphtheria Toxin
CsCl	Caesium Chloride
CSF	Cerebrospinal Fluid
CTAB	Celytrimethylamonium Bromide
ddNTP	Dideoxy-Nucleotide Triphosphate
DE	Detergent Extract
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-Nucleotide Triphophate
DTP	Diphtheria

EDDHA	Ethylenediamine di(o-hydroxyphenylacetic acid)
EDTA	Ethylenediamine Tetra-Acetic Acid
EE	Elugent Extract
EI	Electron Ionisation
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
ET	Electrophoretic Type
EU	Endotoxin Unit
FAB	Fast Atom Bombardment
Fbp	Ferric Binding Protein
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
fM	Femtomole
G	Guanine
GST	Glutathione-S-Transferase
h	Hours
H_2SO_4	Suphuric Acid
HCl	Hydrochloric Acid
HMW	High Molecular Weight Proteins (>65 kDa)
HRP	Horseradish Peroxidase
hTf	Human Transferrin
i.n	Intranasal
i.p	Intraperitoneal
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-Thiogalactopyranoside
kb	Kilobase
kDa	Kilo Dalton

KWC	Killed Whole Cells
L	Litre
L1	Low pool 1 Proteins (<25 kDa)
L2	Low pool 2 Proteins (25-35 kDa)
L3	Low pool 3 Proteins (35-43 kDa)
LAL	Limulus Amoebocyte Lysate
LB	Luria-Bertani
LMW	Low Molecular Weight Proteins (<43 kDa)
LOS	Lipo-Oligosaccharide
LPS	Lipopolysaccharide
M	Moles
m/z	Mass to Charge Ratio
mA	Milliamp
MALDI	Matrix Assisted Laser-Desorption Ionisation
mg	Milligram
$MgSO_4$	Magnesium Sulphate
MHB	Mueller-Hinton Broth
min	Minute
Ml	Millilitre
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Type
mM	Millimole
MMR	Measles, Mumps, Rubella
MMW	Medium Molecular Weight Proteins (43-65 kDa)
MMW+HMW	Medium and High Molecular Weight Proteins (>43 kDa)
MS	Mass Spectroscopy
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometre
Nsp	Neisserial Surface Protein
NZY	New Zealand Broth
°C	Degrees Centigrade
OD	Optical Density
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicle
Opa	Opacity protein
Opc	Opacity protein
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHLS	Public Health Laboratory Service
pM	Picomole
PMSF	Phenylmethyl Sulphonyl Fluoride
Por	Porin
Rf	Relative Mobility
Rmp	Reduction Modifiable Protein
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
RSV	Respiratory Syncitial Virus
SBA	Serum Bactericidal Activity
SDS	Sodium Dodecyl Sulphate

SEAC	Surface Enhanced Affinity Capture
SELDI	Surface Enhanced Laser-Desorption Ionisation
SEND	Surface Enhanced Neat Desorption
Sod	Superoxide Dismutase
T	Thymine
TAE	Tris-Acetate-EDTA
Tbp	Transferrin Binding Protein
TE	Tris-EDTA
Temed	N, N, N', N'-Tetramethylethylenediamine
TIGR	The Institute for Genomic Research
Tm	Melting Temperature
TMBlue	3, 3' 5, 5'-Tetramethyle-Benzidine
ToF	Time of Flight
Tris	Tris-[Hydroxymethyl]-aminomethane
TT	Tetanus Toxoid
Tween	Polyoxyethylene Sorbitan Monolaurate
U	Units
UV	Ultraviolet
V	Volts
v/v	Volume/Volume
VR	Variable Region
w/v	Weight/Volume
wt	Wild-Type
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside
µg	Microgram
µl	Microlitre

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Chapter 1

General Introduction

1.1. *Neisseria meningitidis.*

Neisseria meningitidis is a Gram negative diplococcus that commonly colonises the human nasopharynx (Figure 1.1). Occasionally it invades the host causing meningococcal disease. Invasive *N. meningitidis* are normally encapsulated. The neisseriae grow under aerobic conditions and meningococci grow best on media containing blood, haemin and animal proteins in an atmosphere containing 5% carbon dioxide. With the advent of the *Haemophilus influenzae* type b vaccine, *N. meningitidis* has become the most common cause of bacterial meningitis in the western world.

1.2. The History of Meningococcal Disease.

The symptoms of meningitis have been described as long ago as the time of Hypocrites. In 1805 Vieusseux noted the symptoms of meningitis from an outbreak in Geneva. He described headaches, vomiting, stiffness and rashes on the skin. Matthey, a pathologist at the time, described congestion of the meningeal vessels and blood on the surface of the brain of the dead. *N. meningitidis* was first isolated in the late nineteenth century and soon afterwards meningococci were first isolated from cerebrospinal fluid by lumbar puncture (Cartwright, 1995). In addition to causing meningitis, *N. meningitidis* can be the cause of life-threatening septicaemia. The bacteria grow rapidly in the blood and if untreated, this leads to a cascade of inflammatory responses, circulatory failure and death.

1.3. Pathogenesis by *Neisseria meningitidis.*

The primary stage in infection by *N. meningitidis* is colonisation of the nasopharynx, this involves adherence of the organism to nasopharyngeal cells. Pili on the surface of the meningococcus are involved in primary adhesion to the epithelium. Haemagglutinating strains of *N. meningitidis* (those with pili) adhere better to cells of the nasopharynx than strains that do not haemagglutinate (Salit *et al.*, 1981). Pili bind to receptors on the surface of epithelial cells. Kallstrom *et al.*, (1997) showed that these receptors are CD46 receptors which are found on almost every human cell. Antibodies directed towards

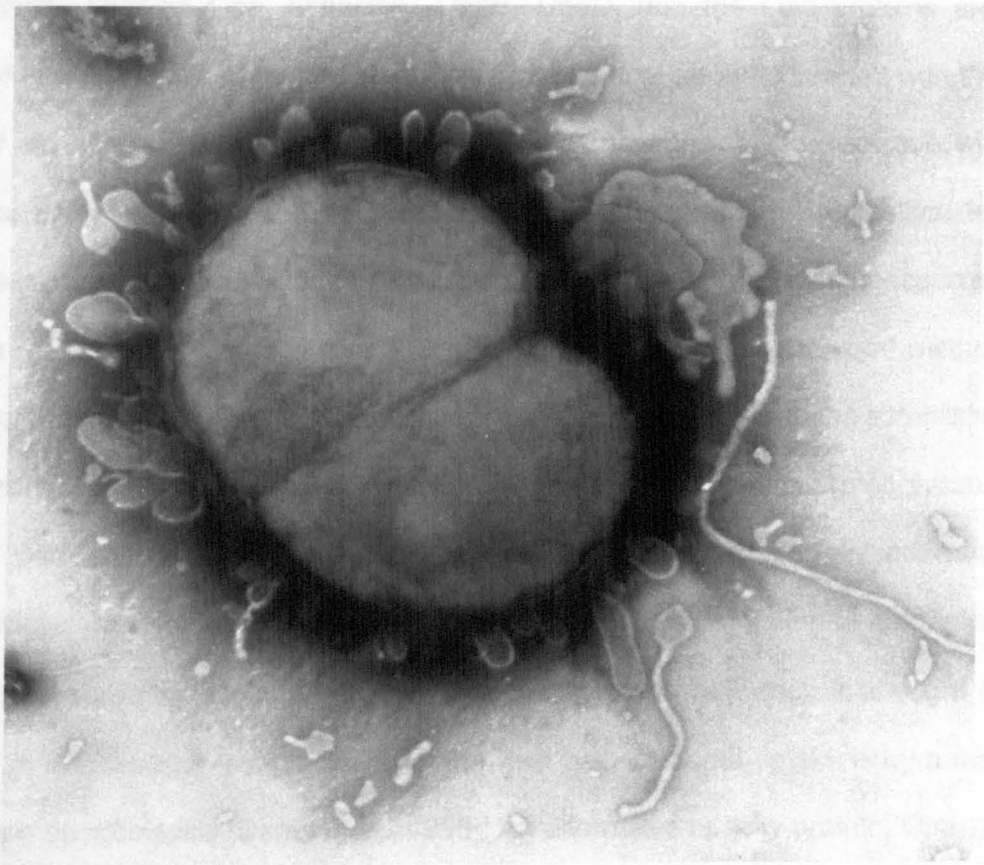

Figure 1.1 Electron micrograph of a meningococcus showing shedding of outer membrane blebs and pili.

CD46 blocked attachment of *N. meningitidis* to Cho cells transfected with human CD46 cDNA *in vitro*. They suggested that bacterial contact between CD46 and pili causes signal transduction which induces internalisation of the bacteria.

The opacity proteins Opa, especially OpaB, OpaD, and the Opc proteins are also involved in colonisation of the host by increasing bacterial ability to interact with the host (Virji *et al.*, 1993). The Opa proteins establish a more intimate association with the epithelial cells than pili alone. Interactions between Opa and the epithelium lead to opsonin-independent uptake of the bacterium. A single strain of meningococcus may express three or four variants of the Opa protein. There may be several methods of uptake of the meningococcus by the host once this association has been established. In Chang cells Opa_{50}, an Opa protein specific for the heparan sulphate proteoglycan, may interact with cell surface associated heparan sulphate, resulting in activation of phosphatidylcholine-dependent phospholipase C resulting in formation of diacylglycerol. This in turn activates sphingomyelinase generating ceramide which is thought to be involved in cytoskeletal reorganisation of the host cell. Bacterial uptake is by a method resembling phagocytosis (Dehio *et al.*, 1998). An alternative opacity protein, Opa_{52}, may initiate internalisation of the meningococcus by interactions with CD66 receptors on the epithelial cell. This results in activation of the *src*-family of non-receptor protein tyrosine kinases, Hck and Fgr causing increased cellular protein tyrosine phosphorylation and activation of Rac1 (a G-protein) which results in cytoskeletal rearrangements and phagocytosis. (Dehio *et al.*, 1998).

Dunn *et al.*, (1995) showed that meningococci are toxic to the epithelium of the nasopharynx and that the level of toxicty corresponded to the adherance of the bacterium to the human epithelial cells. Using polymixin B, a biological inhibitor of LPS they showed that LPS was the cause of the observed toxicity. They suggested that the interaction might aid in breaching the epithelium of the respiratory tract required for meningococcal disease.

Once the meningococcus has adhered to the epithelial cells, IgA protease is produced which reduces local mucosal immune responses by cleaving IgA_1 (Mulks *et al.*, 1978). The production of cilia by the epithelial cells is down-regulated by meningococci producing ciliostatic substances. Non-ciliated epithelial cells engulf meningococci by endocytosis, transporting encapsulated strains to the sub-epithelial layer of cells (Ala'Aldeen, 1995).

Some encapsulated cells are able to resist local immune responses and enter the circulation causing bacteraemia. The meningococcus is protected in the blood stream by its polysaccharide capsule, which includes sialic acid in its composition. Jarvis *et al.*, (1987) showed that in the absence of natural antibody, C3 deposition onto the bacterial surface was increased by enzymic removal of sialic acid from the capsule. This implied that sialic acid was involved in the down-regulation of complement-mediated attack. *N. meningitidis* also sheds blebs of lipopolysaccharide (LPS), pili and other outer membrane components and capsular polysaccharide including sialic acid, to which circulating antibodies are directed. Blebs play an important role in the pathogenesis of meningococcal disease inducing endotoxic shock (Cartwright, 1995). Dunn *et al.*, (1995) showed that viable meningococcal cells and outer membrane vesicles (OMV) had a toxic effect on endothelial cells. Hyperadherant and piliated strains exhibited increased toxicity when compared with non-piliated strains, implying that the level of damage to the endothelium correlated to the ability to bind to the epithelial layer. It is suggested that the synergistic effect of LPS, pili and OMVs cause the haemorrhagic lesions in the skin of an infected person.

Perrin *et al.*, (1999) identified regions of the chromosome of *N. meningitidis* and *N. gonorrhoeae* which are common to pathogenic but not commensal neisseriae. The regions from *N. lactamica* present in the meningococcal and gonococcal genomes were subtracted and both pathogens had sequences in common that were absent from *N. lactamica*. In addition to pilin, IgA protease genes, *porA* and a number of other genes

were present only in pathogenic neisseriae. They suggested that this group of genes were probably involved in common aspects of their life cycle such as colonisation and survival at the port of entry.

1.4. Classification of *N. meningitidis.*

The classification system of *N. meningitidis* is based on antigenic differences in key structures on the surface of the cells such as the capsular polysaccharide (CPS), Class 2/3 outer membrane proteins (PorB), Class 1 outer membrane proteins (PorA) and the lipooligosaccharide (LOS) (Figure 1.2). These structures determine the serogroup, serotype, serosubtype and immunotype, respectively, of different strains. Table 1.1 describes the function and classification of the components of the outer membrane of the meningococcus.

Thirteen serogroups of *N. meningitidis* have been identified due to slight antigenic variations of the CPS (Ala'Aldeen, 1995). Only five serogroups are able to survive in the blood stream, A, B, C, Y and W135. The remainder are killed on entry into the bloodstream. Groups A, B and C are the most common, accounting for approximately ninety percent of cases world-wide. The CPS of some strains, including serogroups B and C, contain sialic acid which prevents complete the assembly of the complement membrane attack complex (Jarvis, 1987). Serogroup B CPS is poorly immunogenic as it resembles a host molecule on host neuronal cell adhesion molecules. Many strains of *N. meningitidis*, particularly those isolated from carriers, do not possess a capsule and these strains are known as non-groupable.

Five classes of outer membrane proteins are expressed constitutively at high levels and classes 1 to 3 are porins which permit the passage of ions across the membrane. Either class 2 or class 3 OMPs, coded for by the *porB* gene, are possessed by all meningococci and, due to the great antigenic heterogeneity of both of these proteins, over twenty serotypes have been identified through the use of monoclonal antibodies

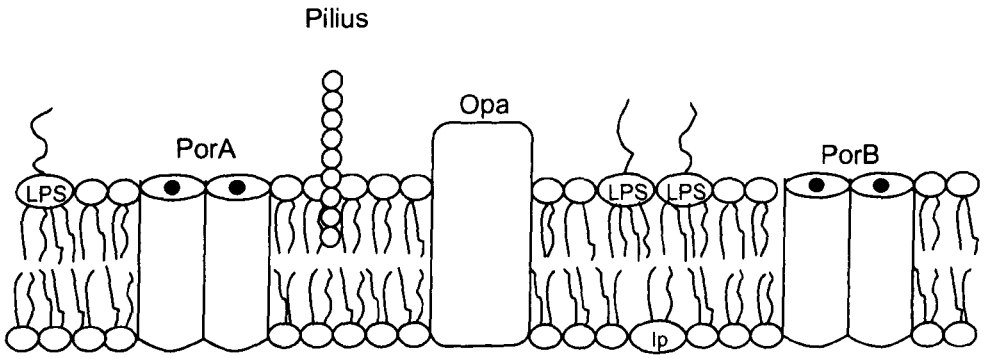

Figure 1.2 Surface structure of the meningococcus. Based on Cartwright *et al.*, 1995. (lp = lipoprotein)

Outer Membrane Component	Molecular Weight	Function	Classification
Polysaccharide capsule		Contains sialic acid which protects the bacterium against the complement membrane attack complex	Serogrouping antigen: 13 serogroups exist
Lipopoly-/Lipooligo-saccharide		Responsible for the toxicity of the meningococcus	Immunotyping antigen: 12 immunotypes exist
Pili		Involved in primary binding of the meningococcus to the epithelium of the nasopharynx	
Opa	26-30kDa	Establish intimate associations with the epithelium of the nasopharynx. Enable opsonin-independent uptake of the meningococcus	Class 5 protein
Opc	25kDa	Establish intimate associations with the epithelium of the nasopharynx	Class 5 protein
PorA	44-47kDa	Permit passage of cations across the membrane	Serosubtyping antigen. Class 1 protein
PorB	37-42kDa	Permit passage of anions across the membrane	Serotyping antigen: over 20 serotypes exist. Class 2/3 protein
Reduction Modifiable Protein (Rmp)	37-42kDa	Unknown	Class 4 protein
Transferrin Binding Protein A (TbpA)	98kDa	Binds host transferrin enabling survival of the meningococcus in the blood	
Transferrin Binding Protein B (TbpB)	65-90kDa	Binds host transferrin enabling survival of the meningococcus in the blood	
Ferric Binding Protein (FbpA)	37kDa	Binds Iron in the periplasm	
Neisserial Surface Protein (NspA)	18-22kDa	Unknown	

Table 1.1 The Outer Membrane Components of *Neisseria meningitidis*; Function, molecular weight and classification. Adapted from Pollard *et al.*, (2001) and Rosenstein *et al.*, (2001)

(Frasch *et al.*, 1985). Heterogeneity is due to surface-exposed variable regions of the proteins. The monoclonal antibodies do not recognise class 2 or 3 proteins possessed by all strains and these are known as non-typeable. Antigenic variations in the class 1 proteins, coded for by the *porA* gene, are used to determine the serosubtype of meningococcal strains (McGuinness *et al.*, 1993). Antigenic diversity results from substitution, deletion and genetic exchange between the surface exposed variable regions VR1 and VR2.

Antigenic heterogeneity of the lipooligosaccharide (LOS), an outer membrane bound glycolipid, between different strains is used to further classify meningococcal cells into immunotypes (Griffiss *et al.*, 1987). Twelve immunotypes exist and individual strains can express more than one immunotype (e.g. L3,7,9). LOS is responsible for many of the toxic effects found in meningococcal disease.

Molecular typing methods are also used for further characterisation of *N. meningitidis*. Multilocus enzyme electrophoresis (MLEE) has been used to divide meningococci into electrophoretic types (ET) based on the electrophoretic mobility of cytoplasmic enzymes. Differences in mobilities of these enzymes are caused by charge differences due to amino acid substitutions in the enzyme peptide sequence (Yakubu *et al.*, 1999). ET5 and ET37 are known as hyperinvasive clones. ET5 causes elevated levels of infection and ET37 is particularly likely to cause localised outbreaks of disease. In Europe, serogroup B disease has been caused mainly by ET5 meningococci and serogroup C disease by ET37 meningococci (Achtman, 1995). Recently *N. meningitidis*, strain W135, ET37, was associated with a disease outbreak (MacLennan *et al.*, 2000).

More recently, multilocus sequence typing (MLST) has been described as a method for characterisation of meningococci (Maiden *et al.*, 1998). This involves comparing the DNA sequences of six housekeeping genes, *aroE*, *pgm*, *adk*, *abcZ*, *pdhC* and *gdh*,

rather than comparing the electrophoretic mobilities of the enzymes they encode. This has been used to define a range of sequence types.

1.5. Epidemiology of Meningococcal Disease.

Meningococcal disease is endemic in all countries, most notably in children less than five years of age. Approximately 10% of the UK population are carriers of *N. meningitidis* at any particular time but annually there are 3-5 disease cases per 100,000 of the population and 10% of these cases are fatal (Cartwright *et al.*, 1995). Between 25% and 40% of young adults are carriers of *N. meningitidis* and most are never affected by the disease. In 1994, Jones *et al.*, (1995) found that 70% of meningococcal disease cases in England and Wales were caused by serogroup B strains and the majority of the remaining cases by serogroup C strains. In the developed world serogroup B meningococcal disease is most common. In the UK, between the years 1996 and 2000, serogroup B disease accounted for 59% of all disease cases, serogroup C for 36% and serogroup W135 for 5% (www.phls.org.uk). Figure 1.3 shows the prevalence of meningococcal disease caused by the various serogroups between 1989 and 2001. The epidemiology of meningococcal disease has changed over the last few years (Memish *et al.*, 2002). Serogroups A, B and C account for 90% of disease cases worldwide. In Europe serogroup C is increasing, serogroup Y is increasing in the USA and Sweden and W135 in Saudi Arabia.

In the 'meningitis belt' of Africa, a region extending the width of Africa from Gambia to Ethiopia, epidemics of serogroup A disease have a regular cyclical pattern of 8 to 14 years (Moore, 1992). Serogroup A disease is rarely associated with developed countries and in Africa, children susceptible to meningococcal disease are older (5-10 years) than in industrialised nations. During a recent epidemic, the percentage of cases of meningitis caused by meningococci approximately doubled and as well as serogroup A disease, W135 disease has emerged in Cameroon, Burkina Faso and Niger (Taha *et al.*, 2002, Fonkoua *et al.*, 2002).

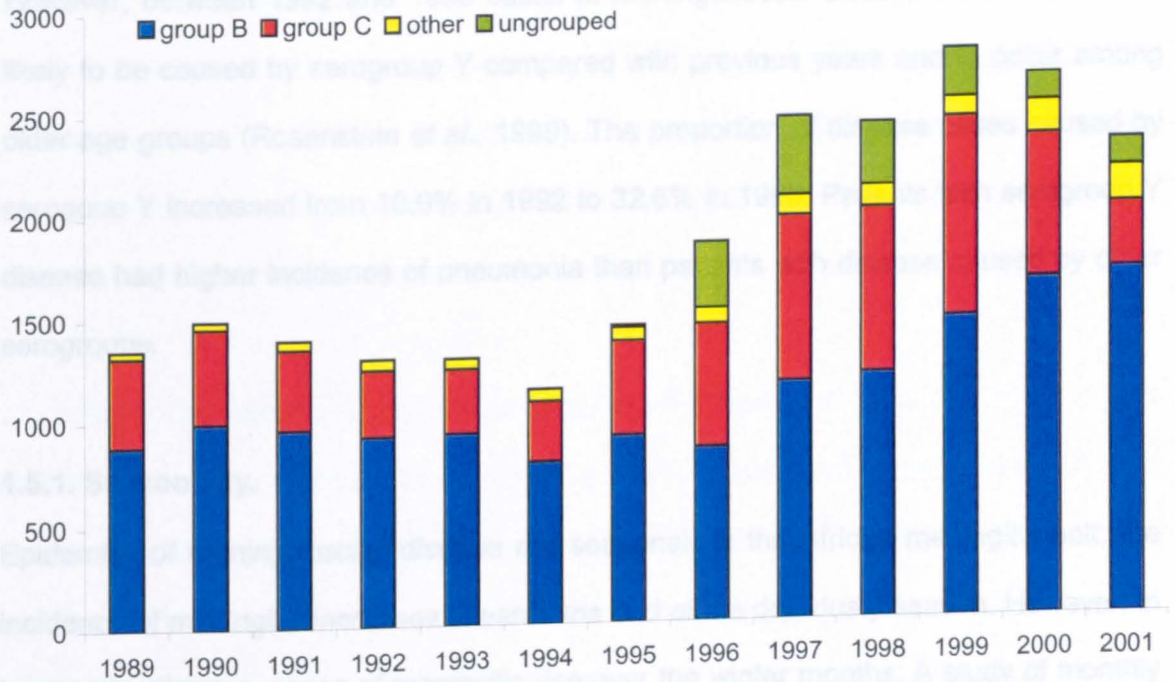

Figure 1.3 Incidence of meningococcal disease in England and Wales between 1989 and 2001. From 1996 data includes PCR confirmed cases in addition to culture confirmed cases. (Data obtained from PHLS Meningococcal Reference Unit)

Pinner *et al.*, (1986) studied the epidemiology of meningococcal disease in the United States. Forty-seven percent of cases of meningococcal disease occurred in those aged less than two years and the incidence of meningococcal disease was higher in the black and hispanic populations. Approximately 48% of disease cases were attributed to serogroup B, 46% to serogroup C, 3% to serogroup W135 and 1% to serogroup Y. An increase is serogroup C disease was observed in 1986 when the study was carried out. However, between 1992 and 1996 cases of meningococcal disease were much more likely to be caused by serogroup Y compared with previous years and to occur among older age groups (Rosenstein *et al.*, 1999). The proportion of disease cases caused by serogoup Y increased from 10.6% in 1992 to 32.6% in 1996. Patients with serogroup Y disease had higher incidence of pneumonia than patients with disease caused by other serogroups.

1.5.1. Seasonality.

Epidemics of meningococcal disease are seasonal. In the African meningitis belt, the incidence of meningitis increases towards the end of the dry, dusty season. However, in temperate climates, cases of meningitis rise over the winter months. A study of monthly meningococcal disease incidence from 1993 to 1994 in England and Wales indicated that cases followed the pattern observed for temperate climates (Jones *et al.*, 1995). It is unknown why seasonality occurs, but it is thought that in the African meningitis belt, dust interferes with mucosal immune responses in the nasopharynx; in areas with temperate climates the observed increase over winter is due to closer personal contact and the increase in catarrhal infections (Cartwright, 1995).

1.5.2. Age Related Incidence.

Meningococcal disease is most prevalent in young children, peaking at about six months of age (Goldschneider *et al.*, 1969). Jones *et al.*, (1994) showed that 54% of isolates in England and Wales were from children aged between 0 and 4 years. Immunity to meningococcal disease is associated with the presence of circulating

bactericidal antibodies, which develop after carriage of different strains of *N. meningitidis*. Young children are unlikely to have been exposed to a large variety of strains and therefore do not possess bactericidal antibodies. A slight peak in the disease occurs in young adults aged between 17 and 18 years (Goldschneider, 1969), corresponding to the high carriage rate observed in this age group (Stuart *et al.*, 1989)

1.6. Host Susceptibility.

Many factors affect the host's susceptibility to the disease. Davies *et al.*, (1996) found that social class, being female and having had rhinorrhoea in the previous month as well as personal and parental smoking had statistically significant associations with carriage of *N. meningitidis* and thus incidence of disease.

Smoking is associated with increased susceptibility, having a damaging effect on the mucosal epithelium of the nasopharynx. In a survey of staff and students at a school in Scotland, 46% of smokers were found to be carriers of *N. meningitidis* compared with 25% of non-smokers. There was found to be no association between passive smoking and carriage (Blackwell *et al.*, 1990). However, Kremastinou *et al.*, (1994) found that children in Greece who were in close contact with smokers had a higher carriage rate of *N. meningitidis* (7.5%) than children whose contacts were non-smokers (3.5%). Riordan *et al.*, (1998) found that although there was a strong association between carriage of *N. meningitidis* and smoking, this was not so for acquisition of meningitis. They suggested that smoking may increase the duration of meningococcal carriage rather than increase the rate of acquisition. El Ahmer *et al.*, (1999) investigated the effects of cigarette smoking on binding of a number of respiratory pathogens, including *N. meningitidis*, to buccal epithelial cells. Greater adherance of each isolate tested was observed for cells from smokers than non-smokers. After treatment of cells from non-smokers with 1/10 dilution of water soluble cigarette smoke extract, binding of the respiratory pathogens increased (however, binding decreased when the cells were treated with neat cigarette smoke extract). They found that the increased binding of the isolates was not due to

enhanced expression of receptors on the host cells but suggested that coating of the mucosal surfaces with components of the cigarette smoke may alter the epithelial cell surface, enhancing binding of bacteria.

Overcrowding, for example of military recruits, also leads to increased susceptibility to meningococcal disease (Riordan *et al.*, 1998). However, Davies *et al.*, (1996) found that overcrowding did not have an effect on carriage in school children in Wales in 1995; there was no difference in overcrowding between meningococcal carriers and non-carriers. Neal *et al.*, (2000) studied the changing carriage rate of meningococci amongst university students over the first term of the year. This was particularly obvious for students living in halls of residence. Carriage rates increased from 6.9% to 23.1% over the first four days. The average carrier rate was 13.9% in October and this increased to 34.2% by December.

People with an impaired immune system are at a greater risk of contracting the disease due to decreased bactericidal antibodies. However, immune impaired people receiving chemotherapy for leukaemia do not have an increased risk of meningitis probably because they possess prior immunity (Cartwright, 1995). Individuals who are deficient in terminal complement component are also more likely to suffer from multiple attacks of meningococcal disease than a healthy individual. Merino *et al.*, (1983) found that 30% of patients who suffered from multiple attacks of meningococcal disease had deficiencies in complement components. Complement deficiency has been found to predispose sufferers to meningitis due to non-groupable meningococci, those strains that are normally vulnerable to bactericidal activity (Fijen *et al.*, 1993).

Respiratory syncitial virus (RSV) has also been suggested to predispose sufferers to meningitis. RSV infection changes the nature of the mucosal surfaces. It was found to enhance expression of CD14, CD15 and CD18, which increases attachment of non-pilate strains (Raza *et al.*, 1999).

Carriage of *N. meningitidis* or *N. lactamica* reduces the risk of developing meningitis due to the development of mucosal immunity and bactericidal antibodies (Gold *et al.*, 1978, Pollard *et al.*, 2001). Assessment of pre- and post- outbreak sera from an outbreak of meningococcal disease at Southampton University in 1997 provided further evidence to support this. Serum from 90% of students (n=76) showed no bactericidal activity towards the disease-causing strain prior to the outbreak. There was low prevalence of *N. lactamica* carriage resulting in a high incidence of invasive disease in those acquiring the outbreak strain (Jones *et al.*, 2000).

1.7. Diagnosis of Meningococcal Disease.

Meningitis is a complication of infection by *N. meningitidis*. The symptoms that are most common include fever, headaches, vomiting, photophobia and sleep verging on a coma. Neurological signs such as seizures are also observed. Initial diagnosis of meningitis requires suspicion that an individual is suffering from the disease, when the appropriate signs are presented. Lumbar puncture is the main method used for confirmation of diagnosis when patients present with some or all of the symptoms described. Cerebro-spinal fluid (CSF) is examined for the presence of polymorphonuclear cells in hundreds to thousands per millilitre, low glucose levels and high levels of protein. Once centrifuged, Gram's stain of the sediment of the CSF and subsequent culturing reveals the presence of the bacterium in approximately 90% of patients (Bohr *et al.*, 1983).

Recently PCR-based techniques have been used to detect meningococci and other bacteria causing meningitis, primarily to decrease the length of time before a diagnosis can be made (24-48 hours). PCR reduces the requirement for culture-based techniques and negates problems with false negative cultures. These are common in developing countries where serogroup A meningococci are the major cause of disease epidemics and where pre-admission antibiotics have been administered. A number of methods for PCR diagnosis have been assessed, for example using primers designed to specifically amplify a fragment of the gene coding for an enzyme involved in the synthesis of the

serogroup A capsular polysaccharide, serogroup A infection can be directly identified (Orvelid *et al.*, 1999). However, these primers do not allow the identification of other meningococcal serogroups. Using primers that amplify a fragment of the *N. meningitidis porA* gene, meningococcal disease can be efficiently diagnosed. Use of an automated DNA analysis system such as DARAS (Tepnel Life Sciences, Manchester, UK) provides accurate detection of PCR products eliminating interpretational problems with conventional agarose gel analysis (Seward and Towner, 2000a). Multiplex PCR has also been investigated which involves simultaneous detection of the sequence coding for bacterial 16s rRNA and the *porA* gene. After completion of PCR, an immunoassay detects positive PCR products. A specific diagnosis of meningococcal disease can be completed within four hours of receipt of the specimen (Seward and Towner, 2000b). However, a TaqMan PCR machine (Applied Biosystems), which enables amplification and detection of amplified DNA to be carried out at the same time (real-time PCR) has been shown by Guiver *et al.*, (2000) to provide sensitive and specific detection of *N. meningitidis* by amplification of the capsular transport gene (*ctrA*). Further investigations have led to the development of multiplex real-time PCR with the TaqMan for the detection of meningitis causing bacteria; *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* (Corless *et al.*, 2001). This involves simultaneous amplification of the *ctrA* gene from *N. meningitidis*, the capsulation gene from *H. influenzae* (*bexA*), and the pneumolysin gene from *S. pneumoniae* (*ply*) for detection of the three main causes of bacterial meningitis. Fluorescently labelled probes are incorporated into the reaction mixture and the TaqMan takes readings of the fluorescence. The primers are highly specific, not amplifying DNA from other bacteria or viral DNA and not reacting with human genomic DNA, but amplifying the DNA from a diverse range of the target bacteria. In the case of *N. meningitidis*, the *ctrA* primers were used to amplify parts of the gene that are highly conserved and common to all meningococci. This technique is used for diagnosis by the Meningococcal Reference Unit, Manchester PHL.

Most patients suffering from meningococcal disease show signs of septicaemia, diagnosed by the presence of a skin rash and blood cultures that are positive for *N. meningitidis*. Three patterns of rash are observed: maculopapular, petechial and necrotic. Maculopapular rash lesions are pink and blanch under pressure. They are usually distributed over the torso. Petechial rash lesions are small and do not blanch under pressure. They are found on the torso and lower limbs and may become confluent and develop into necrotic rash. Frequently patients with septicaemia go into shock due to the circulating endotoxin (Cartwright,1995).

If acute meningococcal disease is left untreated it is fatal. Initial treatment involves administration of intravenous antibiotics such as penicillin G or for individuals who are allergic, cefotaxime or ceftriaxone.

1.8. Meningococcal Vaccines.

Many approaches have been taken towards developing a vaccine which protects against meningococcal disease. Vaccines have been produced which are protective against serogroup A and C strains but none have yet been shown to protect completely against serogroup B disease due to its poorly immunogenic CPS (see section 1.4). The ideal vaccine is one that provides protection and induces cross-reactive antibodies against the five virulent serogroups of meningococcus and the surface proteins of hyperinvasive families, induces T-cell dependent immunity, reducing age dependence and provides good booster responses.

1.8.1. Whole Cell Vaccines.

Whole cell preparations were the first type of vaccine to be developed for meningococcal disease. These were found to have variable efficacy and reactogenicity, so their use was discontinued (Cartwright, 1995).

1.8.2. CPS Vaccines.

CPS has been the main focus for the development of vaccines against meningococcal disease due to the role it plays in protecting *N. meningitidis* from the immune response. Also CPS vaccines have been prepared from *S. pneumoniae* against pneumococcal disease and used for many years (Austran, 1977, Mufson, 2000)

The persistence of serum antibodies to a serogroup A meningococcal CPS vaccine in children vaccinated between the ages of 10 weeks and 19 years were studied in Finland over the period 1975-1979 (Kayhty *et al.*, 1980). Antibodies could be detected for one year post vaccination in children younger than 12 months only after a booster vaccination was given. Antibodies persisted for two years in children aged 12-17 months after a booster vaccination. Children between 18 and 23 months did not receive a booster vaccination and their antibody response declined within one year. A booster vaccination of the serogroup A CPS vaccine was required to maintain antibody titres for more than one year.

Gotshlich *et al.*, (1969) produced the first safe and effective meningococcal vaccine from serogroups A and C CPS. Immunisation with these vaccines induced the production of both haemagglutinating and bactericidal antibodies. During an epidemic of serogroup C disease it was shown that vaccination with the serogroup C CPS significantly reduced acquisition of serogroup C meningococci compared with unvaccinated individuals. The efficacy of a serogroup C CPS vaccine was also assessed in a large trial where approximately 20% of a susceptible population were vaccinated with a 50 μg dose of the vaccine and the efficacy of the vaccine, assessed as acquisition of serogroup C meningococci, was 87% (Artenstein *et al.*, 1970).

The current CPS vaccine, widely used around the world, is a tetravalent vaccine consisting of the CPS of serogroups A, C, W135 and Y. In 1994, vaccination of recruits to the Israel Defence Force with the tetravalent polysaccharide vaccine became routine.

Previously between 1984 and 1994 there were twenty-one cases of meningococcal disease caused by a variety of different serogroups. However, after routine immunisation was introduced, only four cases occurred. Two were due to serogroup C meningococci but occurred in unvaccinated soldiers enlisted before 1994 and the remainder were due to serogroup B, which does not form part of the tetravalent vaccine. The immunogenicity of the tetravalent vaccine was also studied by Cadoz *et al.*, (1985) who found it induced significantly increased levels in bactericidal antibodies directed towards all serogroups encompassed by the vaccine. The tetravalent vaccine does not provide protection against serogroup B disease. Another limitation is age dependant protection; the tetravalent vaccine is not effective in juveniles, the age group at greatest risk. Age dependency was also demonstrated for the serogroup A and C polysaccharide vaccine, used until recently in the UK. Serogroup C specific IgG, tested for by ELISA, and bactericidal antibody titres were significantly lower in vaccinated children less than two years of age than older children (Borrow *et al.*, 2000).

CPS, like other polysaccharides, is a T-cell independent antigen and the immune system of a young child is not developed sufficiently to respond to an antigen of this nature. The protection afforded by this vaccine is also relatively short-lived and a booster response is not normally seen. The vaccine is mainly used to control outbreaks of meningitis that are caused by the serogroups covered by the vaccine.

1.8.3. Conjugated CPS Vaccines.

By conjugation of the CPS to a carrier protein some of the problems encountered by the tetravalent vaccine can be overcome. Conjugation to diphtheria and tetanus toxins has successfully converted the polysaccharide from a T-cell independent antigen to a T-cell dependent antigen (Goldblatt, 1998). Responses in the very young and the length of the immunity are also improved by conjugation to a carrier protein.

Jennings *et al.*, (1981) showed that conjugation of serogroups A and C CPS to tetanus toxoid (TT) converted the antigens into T-cell dependent antigens. The immunogenicity of the two polysaccharides was increased when examined in mice and rabbits compared with the native polysaccharide. Trials involving conjugated serogroup B CPS have been unsuccessful. Conjugation of serogroup B CPS to TT did not increase the immunogenicity of the CPS and no bactericidal activity to the homologous strain was detected in mice or rabbits (Jennings *et al.*, 1981).

Several groups have carried out trials involving a modified serogroup B CPS conjugate. Jennings *et al.*, (1985) originally modified the structure of the serogroup B CPS and conjugated it to tetanus toxoid. Antigenicity of the serogroup B CPS was preserved by maintaining carboxylate and N-carbonyl groups intact; these are required for preservation of CPS conformation. The most successful modification involved the substitution of N-acetylated groups with N-propionylated groups. When conjugated to TT, the modified CPS was found to induce high levels of cross-reactive IgG antibodies specific for serogroup B CPS. The immune response was demonstrated to be T-cell dependent due to the booster response observed. Further work demonstrated that as well as inducing antibodies in mice that were cross-reactive for serogroup B CPS, this conjugate also induced antibodies that were bactericidal for serogroup B *N. meningitidis* (Jennings *et al.*, 1987). Although trials of this vaccine were promising in laboratory animals, there are concerns that use of an immunogenic serogroup B CPS vaccine may induce autoimmune responses in humans due to the resemblance of serogroup B CPS to human antigens. Fusco *et al.*, (1996) produced a conjugate vaccine consisting of the modified serogroup B CPS and a recombinant PorB protein. This vaccine generated elevated bactericidal immune responses over the serogroup B CPS conjugated to TT vaccine against a broad spectrum of serogroup B serotypes and no adverse effects were noted in tests on non-human primates. It was found that the TT conjugate was unable to induce bactericidal activity but the recombinant PorB conjugate generated high levels of bactericidal antibodies after booster vaccinations.

MacDonald *et al.*, (1998) investigated the induction of immunological memory by serogroup C polysaccharide conjugated to CRM_{197} when compared with the plain C polysaccharide vaccine in toddlers aged between 15 and 23 months. CRM_{197} is a non-toxic mutant of diphtheria toxin. Toddlers were given two doses of vaccine, two months apart and, twelve months after the second dose, a follow up dose of plain serogroup C polysaccharide was administered to assess immunological memory. After two doses of the conjugate vaccine, antibody titres were ten-fold greater than for the polysaccharide vaccine and 90% of toddlers produced bactericidal antibody titres of more than 1/8 (the antibody dilution required to kill 50% of bacteria) compared with 32% of those vaccinated with plain polysaccharide. Evidence of induction of a memory response was observed in children administered the conjugate vaccine after twelve months. Hyporesponsiveness was observed with the plain polysaccharide vaccine whilst two fold lower antibody titres were observed following the one year follow-up injection than the control vaccine, a licensed hepatitis B vaccine. After the booster vaccination, bactericidal antibody titres increased dramatically in children given the conjugate vaccine compared to children given the plain polysaccharide vaccine whose bactericidal titre remained the same both before and after the follow-up polysaccharide vaccination.

Three manufacturers competed to produce a meningococcal serogroup C conjugate vaccine. Two of the vaccines are composed of polysaccharide conjugated to CRM_{197} and the third to tetanus toxoid. A CRM_{197} vaccine was assessed by Richmond *et al.*, (1999) and was found to be well tolerated and immunogenic in infants of 2, 3 and 4 months. Bactericidal titres were much higher than those generated by meningococcal polysaccharide vaccines. Richmond *et al.*, (2000) assessed the safety and immunogenicity of the tetanus toxoid conjugate vaccine. The vaccine was well tolerated, the majority of reactions being mild. A greater than 300-fold mean increase in IgG titre was observed in vaccinees when compared to the specific IgG titre prior to vaccination.

The vaccines were made available in the UK in autumn of 1999 and a vaccination program was developed by the Department of Health to introduce the serogroup C vaccine (Donaldson *et al.*, 1999). Table 1.2 shows the schedule for the introduction of this vaccine. The efficacy of the vaccine was monitored in teenagers and toddlers over the first nine months of implementation of the vaccination program (Ramsay *et al.*, 2001). There were 24 laboratory confirmed cases of meningococcal disease in teenagers between 15 and 17 years of age compared to 101 cases during the corresponding period of 1999; a reduction of 76%. Of the 24 cases of meningococcal disease, one occurred ten days post vaccination and the remainder were not vaccinated. In children aged between one and two years, there was a 34% reduction in the disease during the same period. In the unvaccinated group and in people aged between 20 and 44 years, cases of serogroup C disease increased. Four fold increases in serum bactericidal antibody titre were seen in 94% of toddlers (12-14 months) compared to their pre-vaccination titres. Miller *et al.*, (2002) further described the success of the vaccination program on the incidence of meningococcal serogroup C disease. They stated that the total reduction in disease in the vaccinated groups over the period between July 2000 and April 2001 is 81% compared to the same period of 1998/1999.

Concerns have been raised over the possibility of capsule switching by serogroup C meningococci to serogroup B meningococci (Maiden *et al.*, 1999). Selection pressure caused by the serogroup C conjugate vaccine may result in an increase in serogroup B disease against which no vaccine is available. Capsule switching is the result of substitution of the serogroup B *siaD* polysialyltransferase, a capsular biosynthetic gene, with the serogroup C *siaE* polysialyltransferase (Swartley *et al.*, 1997). However, although cases of serogroup B disease increased after the introduction of the serogroup C conjugate vaccine, Ramsay *et al.*, (2001) suggested that as yet there is no evidence of capsule switching occurring.

Phase	Target Group	Dose	Coincident vaccines
1	Babies: 2, 3 and 4 months	3	DTP, Hib, Polio
(autumn 1999)	Babies: 4 and 12 months	2	
	Children: 13 months	1	MMR
	School Children: 15 to 17 years	1	
	Adults: 18 to 19 years on entry to higher education	1*	
2	Children: 14 months to 5 years	1	
(January 2000)	Children: 5 to 14 years	1	
	Adults: 18 to 20 years	1*	

*Vaccination with plain polysaccharide vaccine

Table 1.2 Introduction of the serogroup C conjugate vaccine; the vaccination schedule

1.8.4. Outer Membrane Protein Vaccines.

A vaccine strategy to overcome infection caused by serogroup B strains has been the development of outer membrane vesicle (OMV) vaccines. However these are generally only protective to the homologous strain from which the OMV originated. Studies involving OMV vaccines in Chile, Cuba and Norway have shown that antibodies produced against OMV vaccines are protective against serogroup B strains. The efficacy of an OMV vaccine against serogroup B disease was studied in Norway in a trial involving 171,800 students aged between 13 and 21 years. The vaccine only conferred 57% protection in secondary school children after 29 months and this was insufficient to justify a public vaccination programme (Bjune *et al.*, 1991). More recently phase II trials in two to three year old children of a monovalent serogroup B OMV vaccine in Holland have been described (de Kleijn *et al.*, 2001). The vaccine was administered with either aluminium phosphate or aluminium hydroxide as adjuvants and two vaccination schedules for each vaccine were assessed. The vaccines were either administered three times with three to six weeks between each injection or twice with six to ten weeks between each injection respectively and a booster vaccination was given 20 to 40 weeks after the final vaccination. The vaccines were well tolerated and both vaccines induced bactericidal antibodies but titres were higher when there were three vaccinations before the booster. Vermont *et al.*, (2002) assessed the immune responses of sera from these toddlers. They found that the avidity of antibodies for the homologous OMVs were high after boosting and that there was a strong correlation between bactericidal activity and ELISA titres. IgG1 was the predominant isotype of the antibodies. Cross-reacting IgG3 was also detected but no IgG2 or IgG4 were found.

An OMV vaccine consisting of OMPs from serogroup B meningococci enriched with high molecular weight proteins and blended with serogroup C CPS, called VA-MENGOC-BC, was used to vaccinate secondary school children in Cuba. It was shown to have an efficacy of over 80% and the ability to induce long lasting bactericidal antibodies (Sierra *et al.*, 1990). Based on these results, in 1989 the Cuban Ministry of Public Health carried

out mass vaccination of the population aged less than twenty years and the vaccine was included in the national immunisation programme for all infants. Between the pre-vaccination period (1988) and 1994 the total incidence of meningococcal disease had decreased from 922 cases to 88 cases per 100,000 of the population (Rodriguez *et al.*, 1999). The most marked decrease was in children under three years old. The Cuban vaccine based on OMV from the Cuban epidemic serogroup B meningococcal strain was given to approximately 2.4 million children aged from 3 months to 6 years in 1989 and 1990 in Sao Paulo, Brazil. The vaccine efficacy was estimated as 74% in children aged more than 48 months, 47% in children aged between 24 and 47 months and 37% in children less than 24 months old. Age-dependent differences were observed in the immune responses of the children (de Moraes, 1992). Of 120 children aged between 3 and 23 months, only 25% had a greater than two-fold increase in serum bactericidal titre. Of 80 children aged between 24 and 83 months, 48% had a greater than two-fold increase in bactericidal antibody titre. The rates of serogroup B disease in the children remained approximately equivalent before and after vaccination. It was suggested that the vaccine would be more useful in adults than children if administered routinely in Brazil.

The immunogenicity of the Norwegian and Cuban vaccines were assessed among secondary school children aged 15-20 years in Iceland with the aims of comparing bactericidal activity and antibody titres elicited by the vaccines and the control vaccine (serogroup A/C CPS vaccine); to compare the immune response elicited by two and three doses of the vaccines; and to determine bactericidal activity elicited by the vaccines against heterologous strains. The Cuban vaccine did not produce a significant increase in bactericidal antibody or ELISA titre when compared with the control 12 months after two doses of the vaccine. After vaccination with the Norwegian vaccine, 47% of students had elevated IgG titres. Twelve months after vaccination a third dose of either vaccine provided a significant increase in bactericidal antibody or IgG titre in comparison to the two-dose vaccination schedule. After 20 months however, there was

no significant difference between two and three doses. The vaccines were ineffective against non-vaccine strains and the efficacy of the vaccines in young children was not assessed.

The efficacy of both the Norwegian OMV vaccine and the Cuban OMV vaccine were also compared during an epidemic of serogroup B disease in Chile (Tappero *et al.*, 1999). The vaccines were administered to infants less than one year of age, children aged 2-4 years and adults aged 17-30 years and were administered intramuscularly in a three dose regime, each given two months apart. The control vaccine in two of the groups was the *H. influenzae* type B vaccine and in the adult group the control was aluminium hydroxide adjuvant. No response was observed in infants to heterologous meningococcal strains and only 31-35% of children and 37-60% of adults vaccinated with either vaccine showed an increase in bactericidal antibody titre to homologous strains. It was suggested that neither serogroup B OMV vaccine would provide protection during a heterologous epidemic. OMV vaccines provide best protection in the countries of origin, as they are homologous to the epidemic strain of that country.

During an outbreak in Iquique, Chile, the efficacy of a serogroup B OMP vaccine, purified from the case strain isolated from the outbreak, was assessed in 40,811 volunteers aged 1-21 years. The vaccine strain was genetically similar to the outbreak strain in Norway, Brazil and Cuba and the vaccine consisted of OMPs complexed with serogroup C CPS. During the surveillance period of 20 months post vaccination, there were 18 cases of serogroup B disease and protection was observed only in volunteers aged between 5 and 21 years; the vaccine efficacy for this age group was 70%. However, the overall efficacy of the vaccine was 51%. Although IgG titres were highest amongst the youngest children, the IgG titre declined between 8 weeks and 6 months post vaccination. Bactericidal antibody responses were greatest amongst the older children and an increase in titre was observed over the surveillance period. It was suggested that this increase in bactericidal titre was a result of meningococcal carriage.

The efficacy of the vaccine was high enough for it to be useful in older children and adults during an outbreak. (Boslego *et al.*, 1995)

Research is being carried out into the use of the major OMPs for vaccines and, as most strains of *N. meningitidis* possess class 1 and either class 2 or class 3 OMPs, these have been the main focus of attention. However, due to the considerable heterogeneity between strains of these proteins, vaccines consisting of these proteins are serotype/subtype specific. For example seventy-three different PorA types were found from 281 strains associated with sporadic incidence of meningococcal disease in USA (Sacchi *et al.*, 2000). To overcome the specificity of OMP vaccines, a multivalent vaccine was constructed by insertion of additional *porA* genes into the chromosome of H44/76, deficient in PorB (Van der Ley *et al.*, 1992). Immunisation of mice with OMPs isolated from this strain produced high bactericidal titres for strains with the homologous PorA. Christodoulides *et al.*, (1998) have shown that immunogenicity of PorA is increased when incorporated into liposomes.

A hexavalent PorA vaccine has recently been used to vaccinate infants aged between eight and twelve weeks in clinical trials (Cartwright *et al.*, 1999). OMVs from two isogenic strains each expressing three variant PorA OMPs were prepared and formulated into a vaccine. Four doses were found to induce good immune responses to the homologous six strains from which the PorA originated. Passive protection in rats by monoclonal antibodies raised against class 1, 2 and 3 OMPs of different strains of *N. meningitidis* was assessed (Saukkonen *et al.*, 1989). It was found that monoclonal antibodies to the PorB proteins were not protective but monoclonal antibodies to the PorA proteins were highly protective and bactericidal. Although PorA vaccines are highly immunogenic, disease causing isolates have been obtained which are PorA negative (Van der Ende *et al.*, 1999) thus limiting the potential utility of PorA-mediated immunity in vaccine design. Furthermore, Newcombe *et al.*, (1998) described a serogroup C isolate containing the insertion sequence IS1301 within the VR1 region of *porA*.

Native OMV vaccines have been suggested as an alternative to the OMV vaccines used in Cuba and Norway (Saunders *et al.*, 1999). These consist of high levels of LOS so could not be used as a parenteral vaccine beause of the toxic effect of LOS, but could be administered intranasally. This vaccine was found to produce large bactericidal antibody titres in mice and the advantage of the native vaccine is that the components remain in their native configuration; antigenic epitopes are not disrupted by detergent. Other authors have also suggested administration of OMV vaccines intranasally due to the induction of both mucosal and serum antibodies (Dalseg *et al.*, 1999).

Administration of OMV vaccines intranasally as opposed to subcutaneously has been investigated (Bakke *et al.*, 2001). Significant IgA responses were observed after four weekly doses of the vaccine in mice. Concentrations of IgA were augmented by a further four doses administered one week apart two months later. Intranasal boosting after subcutaneous priming had the same effect as subcutaneous boosting. Serum from mice primed with the OMV vaccine either subcutaneously or intranasally produced the same bactericidal activity. Increased serum bactericidal activity was only observed after boosting subcutaneously. They suggested that priming intranasally and secondary subcutaneous immunisations may not induce immunological tolerance, as antibody resposes were stronger than after priming and boosting subcutaneously. Katial *et al.*, (2002) assessed the safety and immunogenicity of intranasal administration of a native serogroup B OMV vaccine in adults. They showed that the vaccine was well tolerated even though, being native, it contained up to 25% LOS relative to protein. It did not cause nasal inflammatory responses and levels of secretory IgA and IgG were significant, though levels of total serum antibodies were poor. However, in all cases, independent of the route of administration, protection by OMV vaccines is short lived, age-dependent and inconsistent between the different trials (Katial *et al.*, 2002). It is possible that this is due to the variability of OMVs.

Conjugation of *N. meningitidis* serogroup C polysaccharide to serogroup B OMVs has also been suggested as an alternative OMV vaccine providing protection to both serogroup B and C disease (Fukasawa *et al.*, 1999). Using this approach anti-serogroup C polysaccharide IgG titres and serum bactericidal antibody titres against serogroup C meningococci in mouse sera increase eight and 32 fold, respectively, 7 weeks post-primary vaccination compared to vaccination with plain serogroup C polysaccharide. Anti-OMV IgG titres and serum bactericidal antibody titres against serogroup B meningococci in mouse sera showed no significant difference when compared with vaccination with OMV alone and protection experiments were not described.

1.8.5. Live Attenuated Vaccines.

The use of a live attenuated vaccine to protect against serogroup B disease is an approach that has been suggested by Tang *et al.*, (1999). They propose that serogroup B strains attenuated by recombinant DNA techniques and intranasal administration could work well as vaccines against serogroup B disease. A live attenuated vaccine could be multivalent, consisting of several different strains; the strains would retain their native configuration and if administered mucosally would potentially elicit mucosal immune responses. However, there is a concern that the attenuated strains may revert to a pathogenic form causing disease in recipients of the vaccine following gene transfer from other *Neisseria* species present in the nasopharynx.

1.8.6. Other Vaccine Candidates.

Other vaccine candidates being investigated include LOS, transferrin binding proteins (Tbp), neisserial surface protein A (NspA) and proteins identified from the complete genome sequence of *N. meningitidis* strain MC58 (Pizza *et al.*, 2000). LOS has been conjugated to a variety of carrier proteins and its immunogenicity assessed. L2 and L3,7,9 LOS conjugated to TT and OMPs were found to induce IgG1 antibody responses (Verheul *et al.*, 1993). Administration with adjuvants also induced IgG2a and IgG2b responses in mice but the antibodies induced were not bactericidal for serogroup B

meningococci. Poolman *et al.*, (1991) suggested the use of LOS in vaccine formulations due to its limited heterogeneity. However, induction of bactericidal antibodies by LOS is poor. Furthermore, LOS is highly toxic and the cause of septic shock in meningococcal disease patients, thus limiting its use as a vaccine component.

Tbps are involved in the acquisition of iron from the host and vaccination with these proteins may prevent iron uptake by the bacterium. TbpA and TbpB are immunogenic during disease (Gorringe *et al.*, 1995) and antibodies that recognised the TbpA+B complex have been shown to have opsonic activity (Lehmann *et al.*, 1999). Danve *et al.*, (1993) evaluated the purified TbpA+B complex from *N. meningitidis* strain B16B6, as a protective antigen in mice and the bactericidal activity of sera raised against these antigens. TbpA+B were shown to protect mice assessed by both passive and active immunisation. Anti-TbpA+B serum was shown to be bactericidal toward 5 of 10 heterologous strains tested including serogroup A, C and Y strains.

To assess the activity of the Tbps independently, Lissolo *et al.*, (1995) purified TbpA and TbpB and evaluated their capacity to induce antibodies that mediate bactericidal killing of *N. meningitidis*; that inhibit transferrin binding to the proteins; and that inhibit bacterial growth. They demonstrated that anti-TbpB serum exhibited bactericidal activity but anti-TbpA serum did not. Mice immunised with TbpB were protected against meningococcal challenge to a similar extent to mice immunised with the TbpA+B complex. However, the protective efficacy of TbpA was not assessed. TbpA and TbpB-specific antiserum inhibited binding of human transferrin (hTf) to the TbpA+B complex receptor and inhibited growth of the bacterium. West *et al.*, (2001) evaluated recombinant TbpA and TbpB, both isolated in a functional form, individually and in combination for vaccine potential. It was found that recombinant TbpA afforded greater protection than recombinant TbpB to the homologous strain and to 2 heterologous strains tested. However, this protection was observed in mice in the absence of

bactericidal antibodies. In rabbits, however, bactericidal titres were observed but titres were much higher for antiserum raised against TbpB than TbpA.

NspA is conserved among neisseriae. Cadieux *et al.*, (1999) showed sequence comparison of *nspA* from three strains, and NspA antiserum was bactericidal and protective against 14 serologically distinct meningococcal strains. They also showed that 71 meningococcal strains posess the *nspA* gene by DNA hybridisation. However, the value of NspA as a protective antigen against meningococcal disease is disputed. Moe *et al.*, (2001) found that sera raised against NspA from a variety of meningococcal strains show variable passive protection, bactericidal activity and surface binding among a range of strains. It was also shown that NspA is not critical for virulence as a NspA knockout strain is highly virulent in infant rats.

A number of proteins were identified by Pizza *et al.*, (2000) as potential meningococcal vaccine antigens. Five hundred and seventy open reading frames that potentially encoded novel surface exposed or exported proteins were identified from the genome sequence of *N. meningitidis*, strain MC58. Three hundred and fifty genes were successfully cloned into *E. coli* and expressed as either His-tagged or GST fusion proteins. Sera were raised in mice against these proteins and tested by ELISA, bactericidal activity and to assess cell surface exposure of the proteins. Seven proteins were found to be surface expressed, bactericidal and to elicit high IgG titres; two of which induced bactericidal titres similar to that induced by OMV vaccines. Genes homologous to the seven proteins were found in 31 representative strains of *N. meningitidis* suggesting that these proteins could induce immunity against a variety of meningococcal strains.

1.9. *Neisseria lactamica.*

N. lactamica is a commensal Gram negative diplococcus (Figure 1.4). It is very similar to *N. meningitidis* except for its ability to ferment lactose, it is unencapsulated and it is

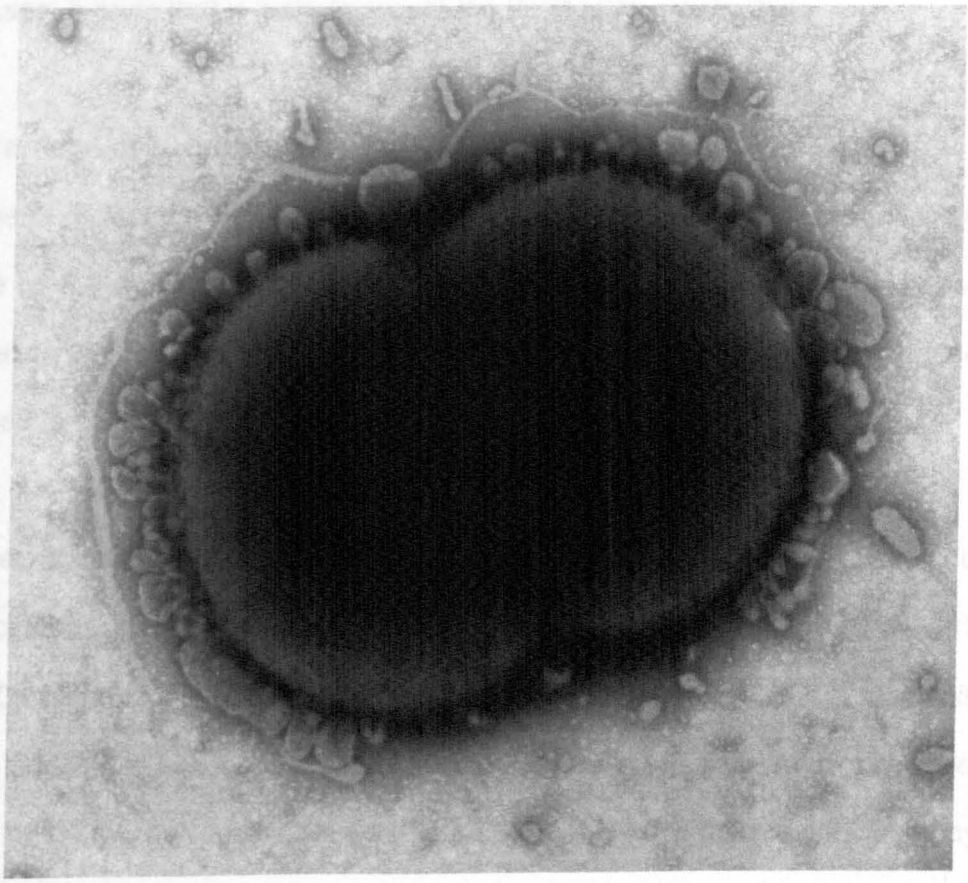

Figure 1.4 Electron micrograph of *N. lactamica* showing shedding of outer membrane blebs.

unable to produce IgA protease, both of which render it non-pathogenic (Muller *et al.*, 1983) though rare cases of encapsulated *N. lactamica* have been reported (Martin *et al.*, 1986). It is able to grow in the media used to select for *N. meningitidis* from clinical isolates. *N. lactamica* does not possess a class 1 OMP (Perrin *et al.*, 1999) but it possesses a porin that is homologous to the PorB of *N. meningitidis.*

1.10. Carriage of *N. lactamica.*

Carriage of *N. lactamica* is thought to be involved in natural immunity to meningococcal disease by the development of bactericidal antibodies. Gold *et al.*, (1978) showed that colonisation by *N. lactamica* led to a fourfold or greater rise in IgG titre to *N. meningitidis* compared to control children who were not colonised.

Nasopharyngeal carriage of *N. meningitidis* and *N. lactamica* was analysed during a survey carried out in Stonehouse, Gloucestershire (Cartwright *et al.*, 1987, Stuart *et al.*, 1989). Carriage of *N. lactamica* was far greater than carriage of *N. meningitidis* in children under the age of five years and there was an inverse relationship between carriage of the two neisserial species (Figure 1.5). This confirmed results found by Gold *et al.*, (1978) in a study of children in Connecticut over the period between 1971 and 1975. Interestingly, male children under the age of fifteen years had a greater carriage rate of *N. lactamica* than their female peers, but the reverse was seen in people over fifteen years of age.

A survey of a community in the Faroe Islands showed that the carriage rate of *N. meningitidis* by *N. lactamica* carriers was approximately a quarter of the rate of non-carriers (Olsen *et al.*, 1991). During a study of meningococcal and *N. lactamica* carriage in Nigeria, carriage rates of *N. lactamica* were higher than carriage rates of *N. meningitidis* (Blakebrough *et al.*, 1982). No sex difference in carriage was observed and the duration of carriage was similar to *N. meninigitidis.* They found that *N. lactamica* carriage declined less rapidly amongst the population when compared to carriage

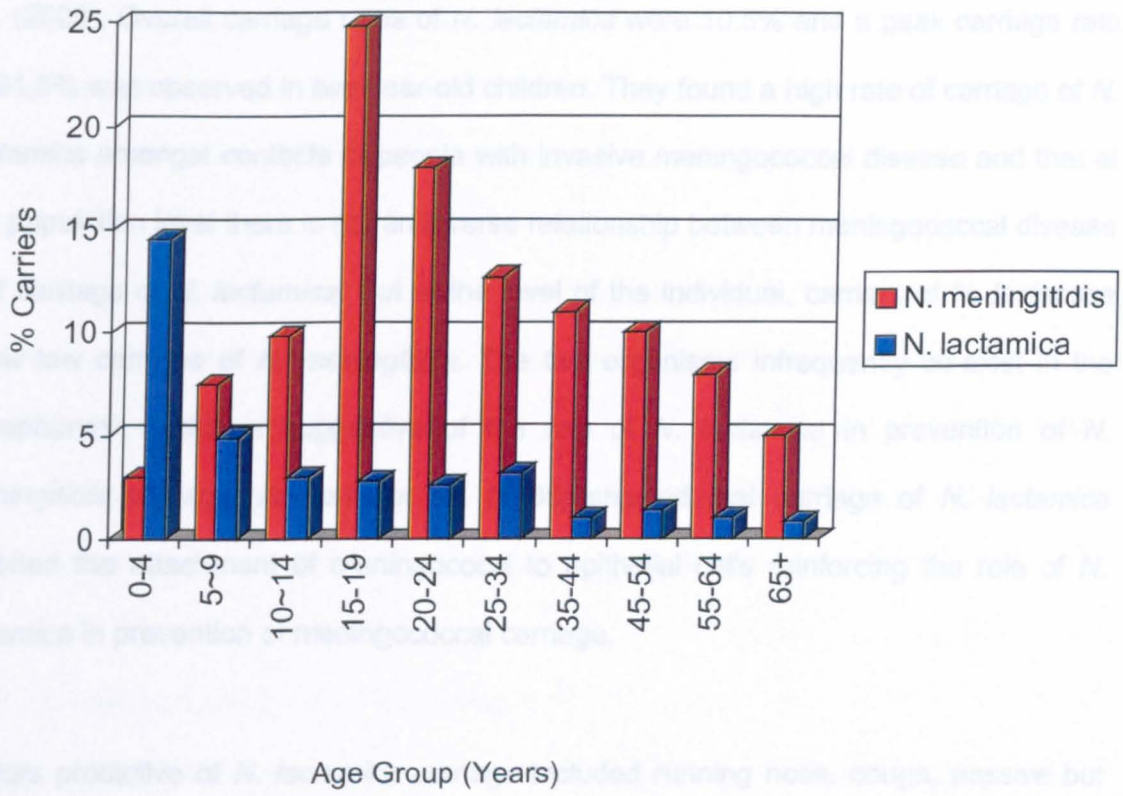

Figure 1.5 Age specific carriage of *N. lactamica* and *N. meningitidis*. Based on Cartwright *et al.*, (1987). The total number of subjects was 5006. Although meningococcal disease is most prevalent in the 0-4 year age group, carriage of *N. meningitidis* is low. Carriage of *N. lactamica* however, is most common in this age group.

studies in the USA. No correlation was observed between meningococcal carriage and *N. lactamica* carriage, however correlation between meningococcal disease and carriage of *N. lactamica* was not examined. Carriage of *N. lactamica* during an epidemic of meningococcal disease in Auckland, New Zealand was investigated by Simmons *et al.*, (2000). Overall carriage rates of *N. lactamica* were 10.5% and a peak carriage rate of 61.5% was observed in two-year-old children. They found a high rate of carriage of *N. lactamica* amongst contacts of people with invasive meningococcal disease and that at the population level there is not an inverse relationship between meningococcal disease and carriage of *N. lactamica*. But at the level of the individual, carriers of *N. lactamica* show low carriage of *N. meningitidis*. The two organisms infrequently co-exist in the nasopharynx, which is suggestive of the role of *N. lactamica* in prevention of *N. meningitidis* carriage. Andrade *et al.*, (1986) showed that carriage of *N. lactamica* inhibited the attachment of meningococci to epithelial cells reinforcing the role of *N. lactamica* in prevention of meningococcal carriage.

Factors predictive of *N. lactamica* carriage included running nose, cough, passive but not personal smoking, age and overcrowding. They suggest that host resistance among individuals not carrying *N. lactamica* has an influence on development of meningococcal disease (Simmons *et al.*, 2000). Kremastinou *et al.*, (1999) found that passive smoking was associated with isolation of *N. lactamica*. They also found that there was a low level of carriage amongst adult males and suggested that the source of *N. lactamica* among children is probably mothers and younger siblings. Using mathematical modelling, Coen *et al.*, (2000) suggest that acquisition of meningococci is inhibited by *N. lactamica*. They state that protection by *N. lactamica* carriage lasts for an average of 4.7 years.

1.11. *N. lactamica* Meningitis and Septicaemia.

In rare cases *N. lactamica* has been found to cause meningitis and septicaemia. A number of authors describe *N. lactamica* infections with symptoms similar to those for meningococcal meningitis; lethargy, fever, irritability, stiffness of the neck and

photophobia (Lauer *et al.*, 1976, Greenberg, 1978, Hansman, 1978, Denning *et al.*, 1991). Septicaemia (Brown *et al.*, 1987, Schifman *et al.*, 1983, Wilson *et al.*, 1976) and acute otitis media (Orden, 1991) have also been reported. A number of these cases were in immunocompromised or debilitated patients. For example one patient had previously suffered skull trauma (Denning *et al.*, 1991) and another was immunosuppressed from chemotherapy following treatment for leukaemia (Schifman *et al.*, 1983). Greenberg (1978) suggested that otitis media might be the initial source of septicaemia or meningitis caused by *N. lactamica*.

Disease caused by other non-meningococcal, non-goncoccal *Neisseriae* has also been reported including *N. mucosa*, *N. subflava* and *N. sicca* (Feder and Garibaldi, 1984). They also found that over a ten-year span only 0.1% of the 8000 cases of neisserial infection was caused by non-meningococcal, non-gonococcal *Neisseriae*.

1.12. Genetic Diversity of *N. lactamica*.

The genetic diversity of *N. meningitidis* has been defined by MLST (Maiden *et al.*, 1998). Studies of the genetic diversity of 26 strains of *N. lactamica* isolated from school children from three different towns were carried out by Alber *et al.*, (2001). The genotypes of these strains were determined using four loci not under immune selection pressure in an analogous fashion to MLST. The four genes; *argF, recA, rho* and the 16S ribosomal gene, were sequenced. Sequencing of these genes indicated a high degree of variability. There were 10 alleles of the 16S ribosomal gene, five alleles of *argF*, nine alleles of *rho* and eight alleles of *recA* among the 26 strains. When taken together a high degree of genetic diversity could be shown for *N. lactamica* by use of the four genes. However, among the 26 strains analysed 17 genotypes were defined. Alber *et al.*, (2001) also showed that recombination occurred frequently amongst *N. lactamica* isolates. It was also unusual to find identical isolates among epidemiologically unrelated hosts. Linz *et al.*, (2000) showed genetic exchange between *N. lactamica*

(and other neisserial commensals) and *N. meningitidis*, indicating that genetic exchange is not species restricted.

1.13. Cross-Reacting *N. lactamica* and *N. meningitidis* Antigens.

Many authors have discussed the presence of cross-reactive antigens between *N. lactamica* and *N. meningitidis*. *N. lactamica* epitopes do not cross-react with *N. meningitidis* serogrouping antibodies and Kim *et al.*, (1989) deduced that cross-reacting antibodies were directed towards the non-capsular antigens. One of 35 *N. lactamica* strains reacted with the serogrouping antigens and this reaction was very weak when assessed by a dot blot assay and this may reflect the lack of specificity of the reagent. However, they found that *N. lactamica* rarely shared epitopes antigenically similar to the PorA or PorB meningococcal OMPs; no cross-reactivity was observed with serotyping or sero-subtyping antibodies. They showed that most *N. lactamica* strains bound the monoclonal antibodies O6B4 or 3F11 which are directed towards L9 LOS. As a result they suggested that LOS epitopes were similar for both *N. lactamica* and *N. meningitidis*.

Cann and Rogers (1989) identified three antigens; proteins of 70, 65 and 15-20kDa common to *N. meningitidis*, *N. lactamica* and *N. polysaccharea* to which cross-reacting antibodies from children's convalescent sera were directed. Bands of 55kDa and 35kDa were specific to *N. meningitidis* and a band of 43kDa was specific to *N. lactamica*. A similar pattern of cross-reactivity was observed for adult convalescent sera. Bactericidal sera were more cross-reactive than non-bactericidal sera. They suggested that the human immune response conferring protection to *N. meningitidis* is to several antigens rather than a single one.

Recently, mouse sera raised against OMVs from both a pathogenic strain of *N. meningitidis* and from *N. lactamica* along with human convalescent sera have been used to identify a number of antigens that are common to both commensal and

pathogenic strains of *Neisseria* (Troncoso *et al.*, 2000). Mouse sera raised against the two types of OMVs showed a high degree of cross-reactivity with the homologous strains. Cross-reactivity profiles of the sera with a number of strains were similar for antigens between 30kDa and 70kDa. Antibodies against *N. lactamica* OMVs were directed towards antigens of 65, 55, 37 and 32kDa from all strains and serum against *N. meningitidis* OMVs were directed towards antigens of 83, 55, 37, 32 and 15kDa. Human convalescent sera reacted with three antigens common to all strains of 65, 55 and 32kDa. Only the 55 and a 32kDa protein were present in all strains of N. *meningitidis* and *N. lactamica* tested. Sanchez *et al.*, (2001) showed that serum raised against the *N. lactamica* OMVs were not bactericidal, but those generated against *N. meningitidis* OMV sera were.

1.14. Study Aims.

It has been suggested that *N. lactamica* is involved in the formation of natural immunity against *N. meningitidis* (Gold *et al.*, 1978, Pollard *et al.*, 2001). The aim of this work was to confirm experimentally that vaccines based on *N. lactamica* can provide protection and to find the components of *N. lactamica* responsible for this protection. To address this aim a number of approaches were taken. Preparative electrophoresis was used to fractionate detergent extracted OMPs from *N. lactamica*. Protection in a mouse i.p. challenge model of meningococcal disease provided by small groups of these protein was assessed and compared with *N. lactamica* OMVs and killed whole cells (KWC) of *N. lactamica*. Two approaches were then taken to identify proteins that react with sera raised against *N. lactamica*. Surface enhanced laser desorption ionisation (SELDI) was used to identify *N. meningitidis* OMPs that cross-react with a variety of *N. lactamica* sera. A phage display genomic library prepared from *N. lactamica* genomic DNA was screened with *N. lactamica* rabbit vaccine antiserum. Inserts from cross-reacting phage were sequenced, their homology with *N. meningitidis* proteins assessed and putative identification of these proteins made. The introduction and methods used will be expanded upon in chapters 3-5 where relevant.

Chapter 2

Materials and Methods

2.1. *N. meningitidis* and *N. lactamica* Strains.

Meningococcal and *N. lactamica* strains used in this study are listed on Table 2.1.

2.2. Routine Growth and Storage of *N. meningitidis*, *N. lactamica* and *E. coli*.

N. meningitidis and *N. lactamica* strains were frozen in Mueller-Hinton broth (MHB) containing 30% (v/v) glycerol, at –70°C, on beads. Strains were recovered by overnight growth on blood agar at 37°C in 5% carbon dioxide (CO_2). The bacterial lawn was resuspended in 5 ml MHB containing 30% glycerol (v/v) and aliquoted into 10 x 500 µl and stored at -70°C. Procedures involving *N. meningitidis* were carried out in a Class III safety cabinet according to local codes of practice.

Bead stocks of *E. coli* were frozen in New Zealand broth (NZY) containing 30% (v/v) glycerol and recovered by overnight growth on either Luria-Bertani (LB) agar or LB supplemented with 12.5 µg/ml tetracycline at 37°C. The bacterial lawn was resuspended in 5 ml NZY containing 30% (v/v) glycerol and aliquoted into 10 x 500 µl and stored at -70°C.

2.3. Growth of *N. meningitidis* and *N. lactamica* under Iron-Limited Conditions.

One hundred millilitres MHB depleted of iron by the addition of ethylenediamine di (o-hydroxy-phenylacetic acid) (EDDHA) (5-10 µg/ml) was inoculated with a 10 µl loopful of agar grown cells and incubated with shaking at 37°C for 18 h. The EDDHA solution was freshly prepared by the addition of EDDHA to 0.36 M NaOH, the pH adjusted to 7.0 with 6 M HCl and the solution filter sterilised (0.2 µm filter).

For the growth of 500 ml culture volumes, a starter culture was prepared by inoculation of 100 ml MHB with a loopful of plate grown cells followed by incubation with shaking at 37°C for 6 h. Five hundred millilitres of MHB depleted of iron with EDDHA was

Strain	Details	Source
Y92-1009	*Neisseria lactamica*	R. Borrow, MRU
K454	B 15 P1,7.16	R. Borrow, MRU
L91 113	B 2b P1.10	R. Borrow, MRU
L2412	A nt	R. Borrow, MRU
L91 543	C 2a P1.2R	R. Borrow, MRU
L93 658	X nt	R. Borrow, MRU
L91 705	B nt P1.6	R. Borrow, MRU
L94-4931	A 4 P1.9R	R. Borrow, MRU
M99 242020	B 2b P1.2, 1.5	R. Borrow, MRU
M99 242 180	B 2b P1.10	R. Borrow, MRU
C11	C	R. Borrow, MRU
JB	B nt	R. Wall, Clinical Research Centre
LV	C nt	R. Wall, Clinical Research Centre
AR	A 4 P1.9	R. Wall, Clinical Research Centre
GN	C nt	R. Wall, Clinical Research Centre
SD	B 15 P1.16	R. Wall, Clinical Research Centre
Z3524	A	M. Achtman, Max-Planck Institute
Z4673	B nt	M. Achtman, Max-Planck Institute
Z4662	B	M. Achtman, Max-Planck Institute
Z8948	C	M. Achtman, Max-Planck Institute
B16B6	B 2a P1.2	A. Schryvers, University of Calgary
B16B6 N96	B 2a P1.2	A. Schryvers, University of Calgary
B16B6 N91	B 2a P1.2	A. Schryvers, University of Calgary
MC58 cap$^-$	B 15 P1, 7.16	S. Kroll, ICSM
MC58 sod C$^-$	B 15 P1, 7.16	S. Kroll, ICSM
H44/76	B 15 P1.7, 16	J. Holst, Oslo
394/98	B 4 P1.4	Chiron

Table 2.1 *N. lactamica and* Meningococcal strains used in this study. All strains listed are meningococci except for Y92-1009 which is a *N. lactamica* strain.

inoculated with a 10 ml starter culture, OD_{650} 1.0, and incubated with shaking at 37°C for 18 h.

2.4. Gel Electrophoresis.

2.4.1. Polyacrylamide Gels.

Samples were analysed on polyacrylamide gels using the method described by Laemmli (1970).

2.4.1.1. Denaturing Gels.

To prepare denaturing resolving gels, Protogel (30% acrylamide/0.8% bisacrylamide (w/v), National Diagnostics) was mixed with resolving gel buffer (Appendix A1) in the appropriate proportions to produce 10, 12 and 15% gels. To 40 ml of the acrylamide mixture, 300 µl of freshly prepared 10% (w/v) ammonium persulphate solutiuon (APS, Sigma-Aldrich) and 30 µl N,N,N',N'-Tetramethylethylenediamine (Temed, Sigma-Aldrich) were added. To prepare the stacking gel, Protogel was mixed with stacking gel buffer (Appendix A1) in the appropriate proportions to produce a 1% gel. To 10 ml, 100 µl APS and 10 µl Temed were added.

2.4.1.2. Non-Denaturing Gels.

Non denaturing gels were prepared in the same way as described for denaturing gels except for the use of different resolving gel buffer and stacking gel buffer (Appendix A1)

2.4.2. SDS-PAGE of Proteins.

Gels were run in a Mighty Small II system (SE250, Hoeffer Scientific instruments). The running tank was filled running buffer (Appendix A1) and the current set at 40 mA per gel.

2.4.3. Preparation of Protein Samples for Electrophoresis.

Protein samples were mixed in the ratio of 1:5 for denaturing gels or 1:1 for non denaturing gels with sample buffer (Appendix A1). Samples mixed with denaturing sample buffer were boiled for 10 min before being loaded onto the gel. Approximately 10 μg/well of protein was loaded onto the gel.

2.4.4. Determination of Molecular Weight on SDS-PAGE.

To determine the molecular weight of protein samples, low range molecular weight standards (Amersham Pharmacia Biotech) were electrophoresed.

2.4.5. Coomassie Staining.

Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (BioRad) in 50% (v/v) methanol and 10% (v/v) acetic acid for a minimum of 1 h. Destaining was with 40% (v/v) methanol solution.

2.4.6. Agarose Gels.

To prepare agarose gels, 0.8% (w/v) agarose was added to desired quantity of TAE (0.04 M tris-acetate, 0.001 M ethylenediamine tetra-acetic acid (EDTA) and heated until the agarose had dissolved. Ethidium bromide was added from a 10 mg/ml stock solution to a final concentration of 0.5 μg/ml once the agarose solution had cooled to 50°C.

2.4.7. Electrophoresis of DNA.

Gels were run at 80-120 V until the dye front had reached the required position.

2.4.8. Preparation of DNA Samples for Electrophoresis.

100-500 ng DNA was mixed in the ratio of 1:6 with sample buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol).

2.4.9. Size Determination of DNA.

To determine the size of the DNA samples, 1 kb DNA ladder (GibcoBRL) was run along side the samples as a standard.

2.4.10. Paragon Serum Protein Electrophoresis.

Electrophoresis of purified serum proteins was carried out using Paragon serum protein electrophoresis system (Beckman-Coulter) as described in the manufacturer's instructions.

2.5. Protein Purification.

2.5.1. Preparation of Detergent Extracted OMPs.

A 500 ml broth culture of *N. lactamica* or *N. meningitidis* was centrifuged at 4000 g for 60 min in an RC3B centifuge with rotor H6000 (Sorval). The supernatant was discarded and the pellet washed with 100 ml phosphate buffered saline (PBS) by centrifugation at 4000 g for 30 min. The supernatant was again discarded and 2 ml PBS containing 0.3% (v/v) Elugent detergent was added for each gram of pellet. The pellet was homogenised using an Ultra-turrax T25 homogeniser (Janice and Kunkel, Germany) and incubated at 37°C with shaking for 20 min. The solution was centrifuged at 14,000 rpm for 10 min and the pellet discarded. To the supernatant 10 mM (w/v) EDTA, 0.5% (w/v) N-lauryl-sarcosine (Sigma-Aldrich) and 0.1% (v/v) of a 10% (w/v) phenylmethyl sulphonyl fluoride (PMSF) solution was added. The supernatant containing extracted OMPs was stored at –20°C.

2.5.2. Separation of Detergent Extract by Preparative Electrophoresis.

Preparative electrophoresis was carried out using the model 419 Prep-Cell (BioRad) as described in the protocol supplied. The gels used were non-denaturing consisting of

either 7% or 12% (v/v) Protogel for separation of OMPs of <100 kDa and <43kDa respectively.

2.5.3. Purification of IgG from Serum.

A Protein G Sepharose Fast Flow gel column (Amersham-Pharmacia) was packed as described by the manufacturer. The serum sample was diluted 1:4 in 20 mM sodium phosphate buffer, pH 7.0 (buffer A) and the column equilibrated with the same buffer. The diluted sample was loaded onto the column and washed through the column with buffer A at a rate of 1 ml/min. The eluate was collected as 5 ml fractions. After 48 ml had washed through the column the buffer was changed for 0.1 M glycine, pH 2.8 (buffer B). This was eluted through the column at 1 ml/min, stripping the column of bound IgG. Twenty-two millilitres of buffer B was washed through the column and the sample was collected in 2 ml fractions into 0.5 ml 1 M Tris, pH 9.0 to neutralise the pH.

2.5.4. Protein Quantification.

Proteins were quantified using the bicinchonic acid (BCA) assay (Sigma) according to the manufacturer's instructions.

2.6. Limulus Amoebocyte Lysate (LAL) Assay.

LAL assay was carried out by the immunoassay group (CAMR) as described by Hochstein (1985).

2.7. Protection Experiments in Mice.

2.7.1. Killed Whole Cell Vaccine.

A 100 ml broth culture was prepared, centrifuged at 10,000 rpm for 30 min and the pellet resuspended in 60 ml PBS containing 600 µl of 40% (v/v) formaldehyde and 0.1% (w/v) thiomersal. The cell suspension was incubated at 4°C for 18 hours. After

homogenising the suspension, it was collected by centrifugation at 3000 rpm for 20 min, the supernatant was discarded and the pellet resuspended to an OD_{650} of 0.1 with sterile PBS containing 25% (v/v) alhydrogel suspension (Superfos, Denmark). Mice were immunised sub-cutaneously (s.c) with 200 µl of resuspended killed whole cell solution.

2.7.2. Protein Vaccine.

Mice were immunised s.c with 10 µg protein in 200 µl 50% (v/v) Freund's complete adjuvant (FCA) for primary vaccinations or Freund's incomplete adjuvant (FIA) for booster vaccinations.

2.7.3. Vaccination Schedule.

Primary vaccinations were administered to groups of 5 female NIH mice (Harlan, UK) aged 6-8 weeks on day 1 of the experiment. The vaccinations were boosted on days 21 and 28 and challenge was on day 35 of the experiment. A group of non-vaccinated mice was included in each protection experiment at each challenge dose. Deaths were monitored for 4 days post infection.

2.7.4. Intraperitoneal (i.p) Infection of Mice.

Mice were challenged i.p with a target dose of 5×10^6 or 1×10^8 CFU of *N. meningitidis* in 500 µl MHB containing 10 mg human transferrin (hTf) as an exogenous iron source. To augment the infection, mice were injected with a further 10 mg hTf 24 h later. Actual doses were calculated by 10^{-4}, 10^{-6} and 10^{-8} dilutions of the challenge doses and colony counts of 100 µl of each dilution after over night growth at 37°C on blood agar.

2.7.5. Production of Sera.

All protection experiments included groups of mice for raising sera. These were immunised with each vaccine but not challenged and sera was taken on day 35 of the experiment.

Rabbit sera were raised following a vaccination schedule similar to that for mice. However each vaccine consisted of 60 μg protein in 2 ml of 25% (v/v) alhydrogel administered at four sites over their backs.

2.7.6. Statistical Analysis of Protective Efficacy.

The statistical significance between the protective efficacy provided by one vaccine to a group of mice and the protective efficacy of another vaccine to a second group of mice was assessed using 2 x 2 contingency tables. The general formula for this is shown on Appendix A2. Significance was assessed at the 95% and 99% confidence levels (Wardlaw, 1985).

2.8. Immunological Techniques.

2.8.1. Enzyme Linked Immunosorbent Assay (ELISA).

The protocols used for ELISA were based on the method described by Abdillahi *et al.* (1987). For whole cell ELISA, microtitre plates (Nunc Maxisorb) were coated as follows. A 100 ml broth culture was prepared and the cells heat killed at 56°C for 30 min. The culture was centrifuged at 2000 g for 20 min and resuspended to an OD_{650} of 0.1 with PBS. A 100 μl volume of the cell suspension was added to each well of a microtitre plate and left to dry for 18 h at 37°C. For protein ELISA, plates were coated with 2 μg/ml protein in coating buffer (1.59 g sodium carbonate, 2.93 g sodium bicarbonate/L) and incubated at 4°C for 18 h. Coated plates were blocked by incubation at room temperature for 1 h with 300 μl PBS per well with 0.1% (v/v) polyoxyethylene-sorbitan

monolaurate (Tween-20, Sigma-Aldrich) (PBS-T) containing 10% (v/v) newborn calf serum. Plates were washed with 0.1% (v/v) PBS-T using a Titertek plate washer (S8/12). Plates were incubated with primary antibody for 1 h, washed as before and incubated for a further hour with species-specific secondary antibody conjugated to horseradish peroxidase (HRP). After washing, the plates were developed with 100 µl of 3,3'5,5'-tetramethyl-benzidine (TMBlue, Intergen) per well for 10 min and the reaction was stopped by the addition of 50 µl of 2 M sulphuric acid (H_2SO_4). Absorbance at 450 nm of each well was determined using the Titertek Multiscan MCC/340 plate reader. Titres were calculated by comparison with the 50% endpoint of a standard pool of all sera assayed.

2.8.2. Western Blotting.

Proteins were transferred to immobilon PVDF membrane (Millipore) by Western blotting using the 2117 Novablot Electrophoretic Transfer Unit (LKB) at a constant current equivalent to 0.8 mA x area of the gel (cm^2) for 1 h. The transfer buffer consisted of 25 mM Tris, 200 mM glycine and 20% (v/v) methanol (pH 8.3). The membrane was blocked with 0.05% (v/v) PBS-T containing 1% (w/v) skimmed milk powder (Marvel) for 1 h. The membrane was washed for 5 min, 3-fold, with 0.05% (v/v) PBS-T, incubated for 1 h in primary antibody dilution, washed again as previously and incubated in species specific HRP conjugated secondary antibody dilution for a further hour. The membrane was finally washed for 5 min, 3-fold, in PBS and developed with 4-chloro-1-naphthol as follows; 400 µl of 2% (w/v) 4-chloro-1-naphthol in methanol was added drop-wise to 40 ml PBS containing 40 µl hydrogen peroxide (H_2O_2) (Sigma-Aldrich).

2.8.3. Serum Bactericidal Assay (SBA).

SBA was performed by a standardised method (Centres for Disease Control, report of the second international workshop on meningococcal immunology and serology, 1992). Briefly blood agar plates were inoculated with 10 fresh, plate grown colonies and

incubated at 37°C for 2-3 h. Cells were resuspended in bactericidal buffer (BB) (20 mg/ml bovine serum albumin and 0.5 U/ml heparin dissolved in Geys balanced salts solution (GibcoBRL)), the OD_{650} adjusted to 0.1 and the suspension diluted by 1/1000 with BB. Twenty microlitres BB was added to the wells of columns 1-11 of a 96 well U-bottom microtitre plate and 10 µl to column 12. Twenty microlitres of heat inactivated serum was diluted using doubling dilutions from column 1-9 and 10 µl to column 12. Ten microlitres of serum containing active complement was then added to columns 1-10 and 10 µl heat inactivated complement to columns 11 and 12. Finally, 10 µl of diluted *N. meningitidis* suspension in BB was added to each well and the plate incubated at 37°C for 1 h. Viable counts were then performed by transferring 10 µl from each well onto brain heart infusion agar (BHI) followed by incubation at 37°C in 5% carbon dioxide (CO_2) overnight. The bactericidal titre is calculated by comparing the viable counts from columns 1-9 with column 11 and analysing for >50% killing. For assays involving serogroup B *N. meningitidis* as the target strain, human complement was used. For assays involving serogroup C *N. meningitidis*, baby rabbit complement was used and heparin was not included in the BB.

2.9. Preparation of Genomic Library.

2.9.1. Preparation of *N. lactamica* Genomic DNA.

100 ml MHB (Oxoid) was inoculated with a loopful of plate grown *N. lactamica* (strain Y92-1009) and incubated with shaking at 37°C for 18 h. The culture was centrifuged at 4000 g and the pellet resuspended in 9.5 ml TE buffer (10 mM tris-[hydroxymethyl]-aminomethane (Tris, Sigma-Aldrich), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), pH 8.0). To this 0.5 ml 10% (w/v) sodium dodecyl sulphate (SDS) (BioRad) and 50 µl 20 mg/ml proteinase K (Sigma-Aldrich) was added to the suspension and this was incubated for 1 h at 37°C. Following incubation 1.8 ml of CTAB buffer (5 M sodium chloride (NaCl) (Sigma-Aldrich) and 1.5 ml celyltrimethylamoniumbromide (CTAB) (Sigma-Aldrich) solution (10% (w/v) CTAB and 0.7 M NaCl)) was added and the

solution incubated at 65°C for 20 min. DNA was extracted form the lysed cells by the addition of an equal volume of chloroform:isoamyl alcohol (Sigma-Aldrich). The solution was centrifuged at 6000 g for 10 min and 0.6 of the total volume of isopropanol was added to the recovered aqueous phase to precipitate the DNA. Precipitated DNA was washed in 1ml 70% (v/v) ethanol by centrifugation at 10,000 g for 5 min, the supernatant discarded and the pellet resuspended in 4ml TE buffer. Exactly 1.075 g/ml caesium chloride (CsCl) (Sigma-Aldrich) and 50 µl of 10 mg/ml ethidium bromide (Sigma) were added and the solution was centrifuged in quick-seal centrifuge tubes at 250,000 g at 15°C for 18 h. The CsCl gradient was visualised under longwave UV and the band removed. Ethidium bromide was removed by sequential extractions with water-saturated butanol. CsCl was removed by precipitation of the DNA with ethanol at 4°C for 15 min followed by centrifugation at 10,000 g for 15 min. The pellet was resuspended in TE buffer for long term storage.

2.9.2. Preparation of Partially Digested Genomic DNA.

Approximately 10 µg genomic DNA was digested as follows; 10 µg genomic DNA, 1 µg BSA, 10 µl NEBuffer 3 (New England Biolabs), 6 µl *MboI* (New England Biolabs) and 63 µl molecular biology grade water (Sigma-Aldrich) were incubated at 37°C for 2 h. The products were run on a 0.8% (w/v) low melting point agarose gel (Sigma-Aldrich). Bands of between 1 and 4 kb were located using longwave UV and cut from the gel. Digested DNA was removed from the gel using the QIAquick Gel Extraction Kit (Qiagen), following the protocol supplied. Extracted DNA was stored at -20°C in TE buffer.

2.9.3. Ligation of DNA and Vector.

To ligate partially digested DNA to the ZAP Express vector (Stratagene) a ligation reaction was set up as follows; 1 µg vector, 0.4 µg digested DNA, 0.5 µl of 10x T4 DNA ligase buffer (New England Biolabs), 2.7 µl molecular biology grade water and 10 units of T4 DNA ligase (New England Biolabs) were incubated at 4°C for 18 h.

2.9.4. Packaging of Vector.

The phage particles were packaged using the Gigapack III Gold packaging extract (Stratagene). Three microlitres ligation reaction was added to the packaging extract and mixed well. The mixture was centrifuged at 6000 g for 5 seconds and incubated at room temperature for 2 h. Five-hundred microlitres SM buffer (5.8 g NaCl, 2 g magnesium sulphate ($MgSO_4.7H_2O$) (Sigma-Aldrich), 50 ml 1M Tris (pH 7.5), 5 ml 2% (w/v) gelatine (Sigma-Aldrich) diluted to 1 L with H_2O) and 20 µl chloroform were added to the mixture, the contents mixed centrifuged briefly and the supernatant stored at 4°C.

2.9.5. Plating Packaged Ligation Product.

E. coli, strain XL1 Blue MRF', was grown overnight on LB agar plate at 37°C for 18 h. A 10 ml LB broth supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose (Sigma) was inoculated with a single colony and incubated with shaking at 37°C for 6 h. The cells were centrifuged at 1000 g for 10 min, the supernatant removed and the pellet resuspended in 10 mM $MgSO_4$. The OD_{600} was adjusted to 0.5 and 2 µl of the final packaged reaction was mixed with 200 µl of XL1 Blue MRF' and incubated with gentle shaking at 37°C for 15 min. Three millilitres NZY top agar at 48°C, 15 µl 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 12.5 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma) were added and the mixture poured immediately onto NZY agar plates and incubated at 30°C for 18 h. Twenty, 9 cm diameter petri dishes (Sterilin) were prepared to cover the entire *N. lactamica* genome. Bacteriophage were recovered by overlaying the plates with 8-10 ml SM buffer and incubating with gentle shaking at 4°C for 18 h. Five percent (v/v) chloroform was added to the bacteriophage suspension and the cell debris removed by centrifugation at 500 g for 10 min. After addition of 0.3% (v/v) chloroform to the supernatant, the library was stored at 4°C.

2.10. Analysis of Genomic Library.

2.10.1. Plaque Lifts.

Three microlitres of the bacteriophage library was plated as described above on to enough NZY agar plates to cover the genome. After incubation at 30°C for 18 h, IPTG soaked nitrocellulose membranes (Amersham Pharmacia Biotech) were applied to the plates and incubated at 30°C for a further 18 h. The membranes were carefully removed and blocked, incubated with antibody and developed with 4-chloro-1-naphthol as described in section 2.8.2 to identify cross-reactive phage.

2.10.2. Plaque Purification.

A plug of agar containing the positive plaque was removed from the plates for each plaque identified. The plugs were placed in 1 ml SM buffer containing 0.5% (v/v) chloroform and incubated at 4°C for 18 h. Phage suspension (10 µl) was plated, lifted and positives identified as previously described. This was carried out for each positive and repeated until the suspensions were pure. Long term storage of phage was at 4°C in SM buffer.

2.10.3. Polymerase Chain Reaction (PCR).

Positive phage were plated as previously described. One plaque was picked for each positive, pipetted into 500 µl molecular biology grade water, vortexed and incubated at 4°C for 18 h to release phage particles from the agar. This was used as the template for PCR. The following reaction mixture was used to amplify *N. lactamica* inserts from positive phage; 1 µl T3 primer (Life Technologies), 1 µl T7 primer (Life Technologies), 1 µl template, 2.5 U Taq DNA polymerase (Roche) , 5 µl 10 x PCR buffer (Roche), 1 µl of 10 mM dNTP (Roche) and 40.5 µl were mixed on ice in a 200 µl PCR tube (Anachem-Scotlab) for each template. The reactions were heated to 94°C for 3 min. Thermal

cycling was repeated 35 times as follows; 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 2.5 min. The reactions were finally incubated at 72°C for 10 min.

2.10.4. Phagemid Excision.

Positive phage were plated as previously described. Individual phage stocks were prepared by transfer of one plaque to 500 µl SM buffer containing 20 µl chloroform, which was then vortexed and incubated at 4°C for 18 h. pBK-CMV phagemids were excised from the ZAP Express vector following the protocol supplied. In brief, 200 µl XL1 Blue MRF' diluted to an OD_{600} of 1.0 was combined with 250 µl phage stock and 1 µl ExAssist helper phage (Stratagene) and incubated at 37°C for 15 min. 3 ml NZY broth was added to each mixture and these were further incubated with gentle shaking at 37°C for 2.5 h and finally heated to 70°C for 20 min and centrifuged at 1000 g for 15 min. The supernatant, consisting of excised pBK-CMV phagemid vector, was decanted and stored at 4°C. This was repeated for each phage stock.

2.10.5. Plating Excised Phagemid.

E. coli, strain XLOLR, was grown in 10 ml NZY broth at 30°C for 18 hours, centrifuged at 1000g for 15 min and the pellet resuspended in 10 mM $MgSO_4$ to obtain OD_{600} of 1. One-hundred microlitres of the phagemid supernatant was mixed with 200 µl resuspended cells and incubated at 37°C for 15 min. Three-hundred microlitres NZY broth was added and the mixture further incubated at 37°C for 45 min. The cell mixture (200 µl) was ten plated on LB agar supplemented with 50 µg/ml kanamycin (Sigma) and incubated at 37°C for 18 h.

2.10.6. Phagemid Purification.

Phagemids were purified using Wizard *Plus* Minipreps (Promega) following the protocol supplied.

2.10.7. Sequencing.

Thirty microlitres of approximately 100 pM/μl purified phagemid stocks were sequenced by Cytomyx (Cambridge) initially using T3 and T7 primers and subsequently by primer walking. New primers were designed from the sequences produced using PrimerSelect (DNAStar) and synthesised by Invitrogen.

2.11. Surface Enhanced Laser-Desorption Ionisation (SELDI).

2.11.1. Coating of Dynabeads with *N. lactamica* IgG and *N. meningitidis* Protein Pools.

Two-hundred and fifty microlitres magnetic tosyl-activated dynabeads were placed into a 1.5 ml tube and the beads retained using a magnet. The solution was removed and the beads were resuspended with mixing for 2 min in 250 μl 0.1 M borate buffer (pH 9.5). This was repeated twice then the buffer was removed and the beads resuspended in 500 μl borate buffer containing 30 μg IgG purified from mouse *N. lactamica* antisera. The beads were incubated for 18 h at 37°C with slow tilt rotation. The beads were blocked by resuspension in 500 μl PBS containing 0.1% (w/v) bovine serum albumin (BSA). The wash was repeated twice then the solution was removed and replaced with 0.2 M Tris (pH 8.5) containing 0.1% (w/v) BSA and incubated for 4 hours at 37°C with slow tilt rotation. The solution was removed and the beads resuspended in 500 μl PBS containing 0.1% (w/v) BSA. The beads were washed in 500 μl PBS containing 0.5% (v/v) Triton-X100, resuspended in 500 μl PBS containing 0.1% (w/v) BSA and finally resuspended in 100 μl PBS containing 0.1% (w/v) BSA. Ten microlitres of the IgG coated bead solution was incubated with 50 μg of *N. meningitidis* OMP pool for 4 h with slow tilt rotation. The supernatant was removed and the beads washed with sterile water or PBS containing 0.5% (v/v) Triton X100 for 5 min in triplicate. Washed beads were resuspended in 10 μl sterile water for analysis. Beads coated with normal rabbit IgG and incubated with *N. meningitidis* OMPs were used as controls.

2.11.2. Analysis of Beads.

Two microlitres of bead solution were placed onto 1 μl 50% (v/v) acetonitrile on each spot of H4 chips. These were left to dry and covered with 0.7 μl of a 10 mg/ml solution of sinapinic acid in 0.25% (v/v) trifluoric acid and 50% (v/v) acetonitile. Proteins bound by the IgG were desorbed from the surface of the beads by the laser and analysed in a time-of-flight mass spectrometer. This produces an accurate mass for each protein species desorbed.

2.11.3. Calibration of the Apparatus.

Calibration was carried out as described in the Ciphergen handbook.

Chapter 3

Separation of and Protection by *Neisseria lactamica* Outer Membrane Proteins

3.1. Introduction.

Vaccines based on outer membrane proteins are a promising approach to prevention of meningococcal disease. Bactericidal antibodies have been shown to be directed against the non-capsular components of the meningococcus (Kim *et al.*, 1989). Outer membrane vesicles, consisting largely of OMPs, are protective in humans, and high molecular weight OMPs (Sierra *et al.*, 1990, Bjune *et al.*, 1991) have been shown to induce bactericidal antibodies in humans. A number of authors have also described the vaccine potential of the OMP PorA (Van der Ley *et al.*, 1992, Cartwright *et al.*, 1999, Saukkonen *et al.*, 1989). Human convalescent sera is cross-reactive with outer membrane proteins from both *N. meningitidis* and *N. lactamica* (Cann and Rogers, 1998, Troncoso *et al.*, 2000). This indicates that the *N. lactamica* proteins may be able to induce protective antibodies against *N. meningitidis*. It has been suggested that *N. lactamica* may be involved in eliciting natural immunity to meningococcal disease. Gold *et al.*, (1978) showed that carriers of *N. lactamica* had greater antibody titres to meningococci than non-carriers and Olsen *et al.*, (1991) found that people colonised with *N. lactamica* had a quarter of the meningococcal carriage rate of non-carriers than *N. lactamica*. An inverse relationship between *N. lactamica* carriage and meningococcal disease has been suggested (Coen *et al.*, 2000). Thus OMPs from *N. lactamica* may be an ideal choice for a meningococcal vaccine protecting against a variety of serogroups and serotypes. This chapter describes separation of *N. lactamica* OMPs by preparative electrophoresis and their immunogenicity and protective efficacy in mice against lethal challenge with *N. meningitidis*.

3.1.1. Protein Separation Techniques.

A number of techniques can be used for the separation of protein mixtures and the purification of proteins from complex mixtures. The majority of these involve some form of chromatography. Column chromatography involves either separation by size or adsorption of proteins to a solid matrix and the removal of the proteins with a change in buffer. Separation of the individual protein components occurs because of their differing

adsorption behaviour (Scopes, 1996). The least adsorbed proteins pass through the column quickly. However, this is not simple as often proteins either do not stick at all or they bind so tightly that they cannot be removed. Changes in buffer properties are often required throughout the course of the chromatography.

Different sorts of chromatography include ion exchange chromatography, hydrophobic chromatography, affinity chromatography, immobilised metal affinity chromatography, and gel filtration. These exploit the properties of the proteins to be separated or purified. For ion exchange chromatography proteins are bound by and removed from the column due to changes in the buffer pH. Similarly changing salt concentrations in the buffers facilitate binding and removal of proteins from hydrophobic columns. Affinity chromatography and immobilised metal affinity chromatography involve binding of the protein to an immobilised ligand or metal ion on the matrix of the column. Gel filtration involves separation of proteins by size as the matrix slows the passage of proteins, the smaller proteins being retained for longer within the matrix.

Equally, proteins can be separated on a size basis by preparative electrophoresis. However, unlike gel filtration, smaller proteins are eluted before the larger ones.

3.1.2. Preparative Electrophoresis.

Preparative electrophoresis involves the separation of mixtures of proteins by gel electrophoresis and collection of the proteins eluted from the gel. Any molecule that can be resolved in a conventional slab gel can be purified using continuous elution electrophoresis. The model 491 Prep Cell (BioRad) can be used to isolate proteins from crude extracts or from partially purified samples and liquid fractions are eluted. The Prep Cell uses conventional polyacrylamide gel electrophoresis (PAGE) and buffer systems to separate proteins. Depending on the gels and buffers used, proteins can be separated in either a native or denatured form. The advantage of using a native system

over a SDS-PAGE is that proteins are not only separated on a basis of molecular weight but also charge.

The Prep Cell consists of many components (Figure 3.1). It has two buffer chambers; the lower buffer chamber holds the electrophoresis running buffer and the upper chamber elution buffer. Buffer runs through a cooling core which cools the gel during electrophoresis. This ensures that proteins migrate in compact parallel bands through the gel. The gel is cast in a gel tube and is in contact with an elution frit and dialysis membrane. The dialysis membrane reduces loss of protein from the gel during elution. Elution buffer continuously flows to the perimeter of the elution frit, is drawn to the centre of the frit and passes through an elution tube in the centre of the cooling core and pumped out to a fraction collector. Once bands of protein migrate from the gel they enter the centre of the frit. The flow of the elution buffer overcomes the downward force of electrophoresis the protein bands are eluted with the buffer.

Three variables are considered when optimising the resolution of the protein bands. These are the gel pore size, the length of the gel and the diameter of the gel. The optimum pore size of the gel depends on the molecular weights of proteins of interest. If specific proteins are to be purified, the optimum pore size is that which results in a relative mobility (Rf) the protein of between 0.55 and 0.6.

$$Rf = \frac{\text{Distance that the protein of interest migrated on a slab gel}}{\text{Distance that the ion front migrated}}$$

Generally however, the higher the molecular weight of the protein of interest the lower the percentage of polyacrylamide required in the gel. Gel length increases the resolution between proteins. Small differences in the molecular weights of proteins require longer gels to resolve the proteins, but a disadvantage of increasing the gel length is increased diffusion of the proteins from the gel. Two different sized gel tubes are available for

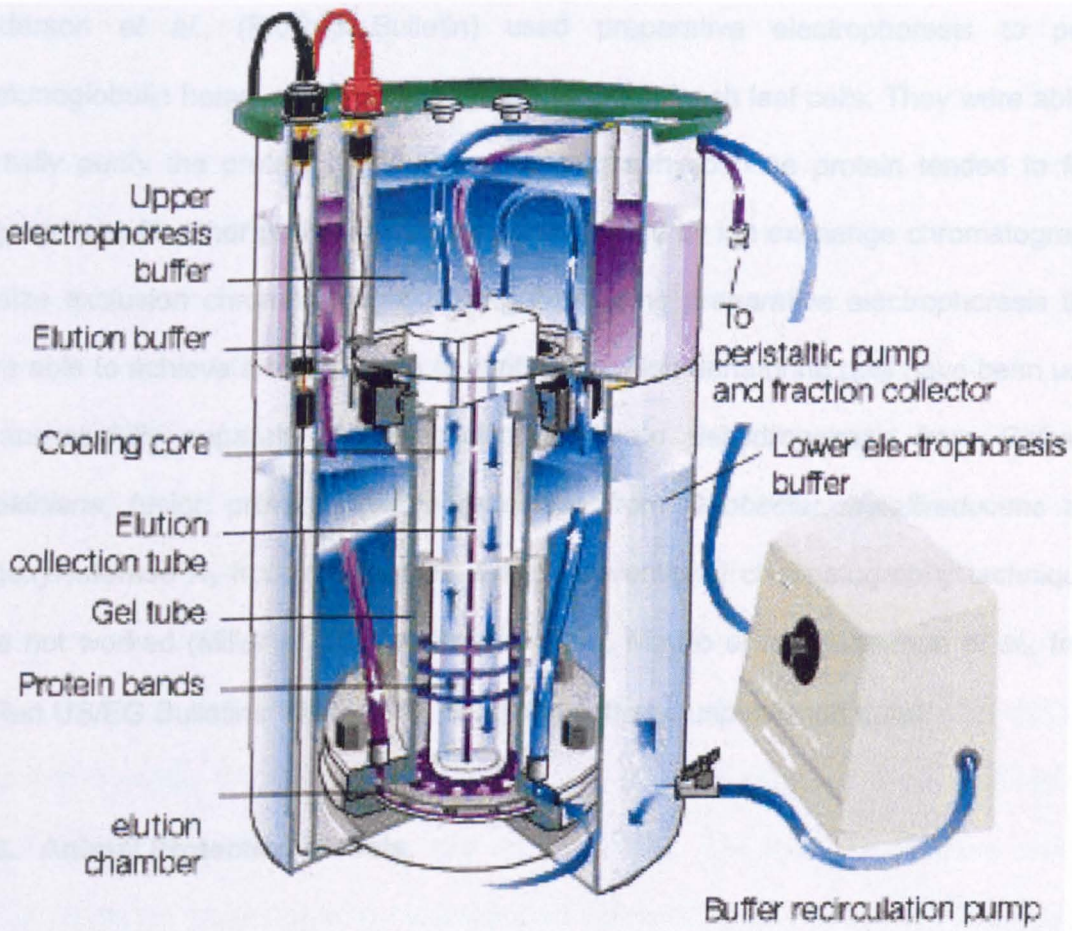

Figure 3.1 Schematic of the Model 491 Prep Cell. Proteins are electrophoresed through the cylindrical gel matrix where they separate into ring shaped bands. Individual bands migrate off the bottom of the gel and pass into an elution frit in the elution chamber. A dialysis membrane traps proteins in the elution frit. As individual bands exit the gel, they are drawn to the centre of the frit, out through the collection tube to a peristaltic pump, which drives separated molecules on to a fraction collector. (Taken from www.bio-rad.co.uk)

preparative electrophoresis and the larger of the two allows the maintenance of compact bands within the gel due to the increased surface area of the gel.

A number of authors have described the use of the prep-cell for separating proteins. Anderson *et al.*, (BioRad Bulletin) used preparative electrophoresis to purify immunoglobulin heavy chain binding proteins from spinach leaf cells. They were able to partially purify the protein by affinity chromatography but the protein tended to form aggregates with other proteins when being separated by ion exchange chromatography or size exclusion chromatography. Using denaturing preparative electrophoresis they were able to achieve a high degree of purification. Non-denaturing gels have been used to successfully separate NADP-specific glutamate dehydrogenase from *Chlorella sorokiniana*, fusion proteins, nitrate reductase from *Geobacter metallireducens* and carboxylesterase A_2 from mosquitoes where conventional chromatography techniques have not worked (Miller *et al.*, Vanderspek *et al.*, Murillo *et al.*, Ketterman *et al.*, from BioRad US/EG Bulletins 1987, 1844, 1839 respectively, unpublished data).

3.1.3. Animal Protection Models.

A number of different animal models have been assessed for the study of meningococcal infection and protection of antigens against meningococcal disease. Animal species used in protection models include monkeys, rabbits, rats, mice, guinea pigs and chicken embryos. However, none of these provide the ideal infection model. Branham and Lillie (1932) produced clinical and pathological meningitis in rabbits but a large dose had to be administered, the clinical effects were variable and meningitis was only observed in 39% of rabbits. Branham *et al.*, (1937) went on to repeat this work in guinea pigs and in this model infection generally remained localised but meningococci were rarely cultured from blood. A guinea pig subcutaneous implant model was described by Frasch *et al* (1978) and smaller inocula of *N. meningitidis* were required to establish localised infection. Passive protection by meningococcal antisera was

assessed in a chicken embryo model (Frasch *et al.*, 1976). The chicken embryo was chosen because, like man, meningococcal infection caused the development of lesions. However they do not posses an active complement system. Miller (1933) discussed the large doses of even the most virulent strains required before guinea pigs and mice succumb to intraperitoneal (i.p.) infection. He suggested that such heavy inoculations might in fact cause intoxication of the animal rather than meningococcal disease. However, supplementary mucin produced fatal infection in mice with very few organisms (Miller *et al.*, 1935). Mucin provides iron in an iron-depleted environment for the growth of *N. meningitidis*. Iron dextran (Holbein *et al.*, 1980) and human transferrin (hTf) (Schryvers *et al.*, 1989) can be used instead of mucin with the same effect. Branham *et al.*, (1940) used a similar model to show that passive protection could be achieved by i.p. administration of serum. Saukkonen *et al.*, (1987 and 1988) developed an infant rat model for testing the passive protection against meningococcal infection of anti-meningococcal antibodies. They showed that monoclonal antibodies to PorA and to capsular polysaccharide were both protective against meningococcal infection. Observed mortality showed good correlation with bacterial load and a clear difference was observed between protection and no protection. The majority of these animal models utilise i.p. challenge with meningococci which does not mimic the natural route of infection in man.

MacKinnon *et al.*, (1992) investigated intranasal (i.n.) infection in a mouse meningococcal challenge model. They tested two inoculum volumes, 3.5 µl or 10 µl, and found that lung colonisation was achieved with a larger inoculum and the fatality rate was greater in these mice. Unlike infection in man, bacteraemia was only observed after colonisation of the lungs. Also the model uses infant mice so cannot be used for protection following active immunisation. In all infection models an exogenous source of iron is required (Arko, 1989). Martin *et al.*, (1962) showed that iron enhanced infection by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in rats and mice. Unsaturated transferrin, an iron binding protein in humans, inhibited the infection to a small extent.

MacKinnon *et al.*, (1992) tested two iron sources, iron dextran and saturated hTf. These supported infection to the same extent but hTf was more effective, as a smaller amount of iron was required to support equivalent infection.

Of the models described, Gorringe *et al.*, (2001) suggests that the most versatile model for meningococcal infection, is the mouse i.p. model. Although the symptoms of meningococcal infection are not the same in mice, the mouse immune system functions in many respects like that of humans and protection following active immunisation can be assessed in this model.

None of the models described provide the ideal meningococcal infection model. For the purposes of protection studies described in this chapter, the mouse i.p. infection model is considered the best available. Exogenous iron in the form of hTf is administered for infection to be maintained. Symptoms of meningitis are not observed but challenge with meningococci causes lethal bacteraemia. This model can be used to assess the protective efficacy of meningococcal vaccines and the immune responses to the vaccines can be investigated.

3.2. Results

3.2.1. Separation of *N. lactamica* Outer Membrane Proteins

OMPs of *N. lactamica* strain Y92-1009 were extracted using 0.3% (v/v) Elugent. The extracted OMPs were separated by preparative electrophoresis using the Prep-Cell model 419 (BioRad). A 7% acrylamide native gel was found to provide good separation of the extracted OMPs (Figure 3.2). Fractions were pooled into three groups based on approximate molecular weight; less than 43 kDa (LMW), 43-65 kDa (MMW) and greater than 65 kDa (HMW) (Figure 3.3). The pooled proteins were concentrated by freeze-drying and used to immunise mice and to assess protection following meningococcal challenge.

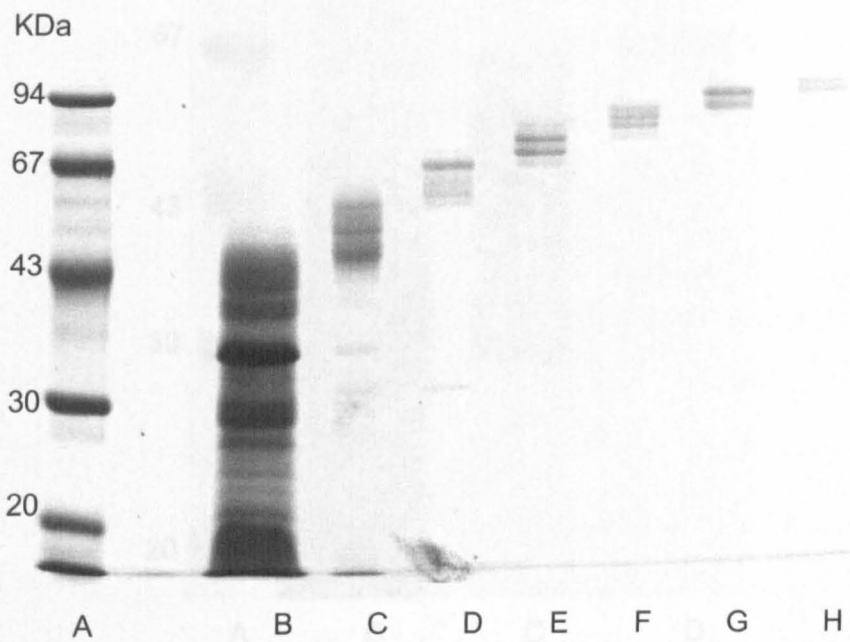

Figure 3.2 *N. lactamica* detergent extracted OMPs separated by preparative elecrophoresis. The molecular weight markers are shown in lane A. Increasing sized OMPs, separated by preparative electrophoresis, are shown in lanes B-H.

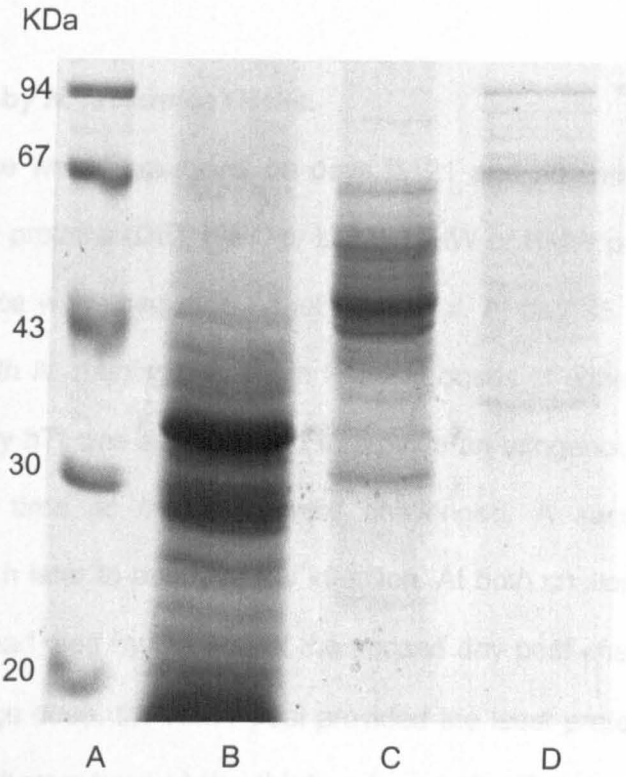

Figure 3.3 *N. lactamica* OMPs separated by preparative electrophoresis were pooled into three groups of proteins. The molecular weight marker is shown on lane A. Lane B shows the LMW protein pool consisting of proteins of approximately <43 kDa. Lane C shows the MMW protein pool consisting of proteins of approximately 43-67 kDa. Lane D shows the HMW protein pool consisting of proteins that are mostly >67 kDa.

The LMW proteins were further separated by preparative electrophoresis using a 12% acrylamide native gel. Once separated, the proteins were again pooled into three groups and concentrated by freeze-drying. The three pools consisted of proteins of approximately less than 25kDa (L1), 25-35kDa (L2) and 35-43kDa (L3) (Figure 3.4).

3.2.2. Protection by *N. lactamica* OMPs.

Groups of five mice were immunised on days 0, 21 and 28 with either *N. lactamica* detergent extracted proteins (DE), KWC or LMW, MMW or HMW protein pools. A group of unvaccinated mice was used as a negative control. At day 35 the immunised mice were challenged with *N. meningitidis* strain K454 at doses of either 2×10^7 or 6×10^8 CFU. Supplementary hTf was administered to provide an exogenous iron source to the mice at the same time as the mice were challenged. A second hTf dose was administered i.p. 24 h later to augment the infection. At both challenge doses all of the unvaccinated mice had died by the end of the second day post challenge (Figure 3.5). At the lower challenge dose, the HMW pool provided the least protection with only one mouse surviving challenge beyond the third and up to the fourth day post challenge. Both the MMW pool and the detergent extract provided good protection. In both cases, challenge was fatal to one mouse by the second day but 4 mice from each group had survived at the end of the experiment. The LMW protein pool provided the best protection with all mice surviving challenge by strain K454. At the lower challenge dose survival was significant, both at the point when there were no survivors in the unvaccinated group, and at the end of the experiment for mice vaccinated with the detergent extract, LMW and MMW ($P=2.3\%$, 0.4% and 2.3% respectively). KWC and HMW provided no protection ($P>5\%$). At the higher challenge dose only mice vaccinated with LMW proteins survived the duration of the experiment. Of this group of mice the survival rate was 60%. Only protection by LMW was significant at the point when there were no survivors in the unvaccinated group ($P=2.3\%$). Protective efficacy of the LMW group was assessed in larger groups of mice (Figure 3.6). At the low challenge dose (5×10^6 CFU) protection was observed in 14/20 mice by the end of the

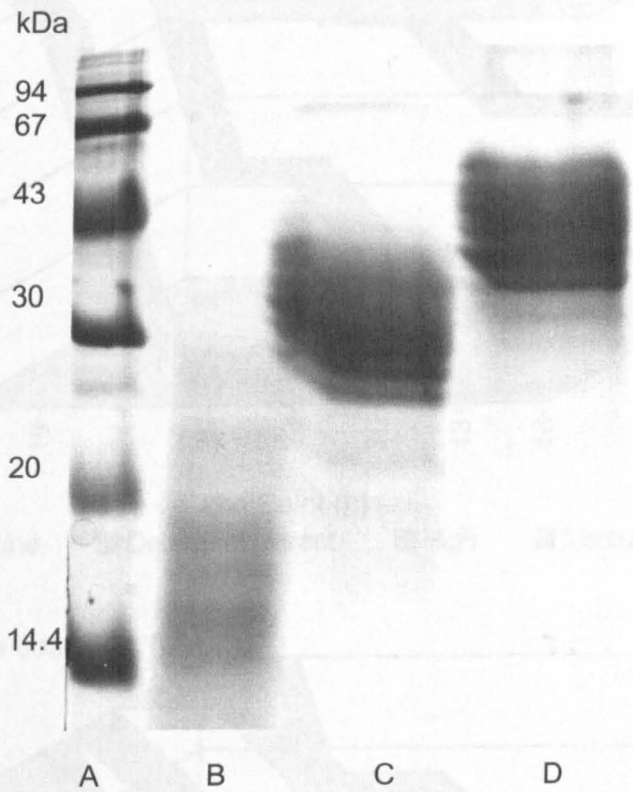

Figure 3.4 *N. lactamica* LMW OMPs separated by preparative electrophoresis were pooled into three groups of proteins. The molecular weight marker is shown on lane A. Lane B shows the L1 protein pool consisting of proteins <25 kDa. Lane C shows the L2 protein pool consisting of proteins 25-35 kDa. Lane D shows the L3 protein pool consisting of proteins 35-43 kDa.

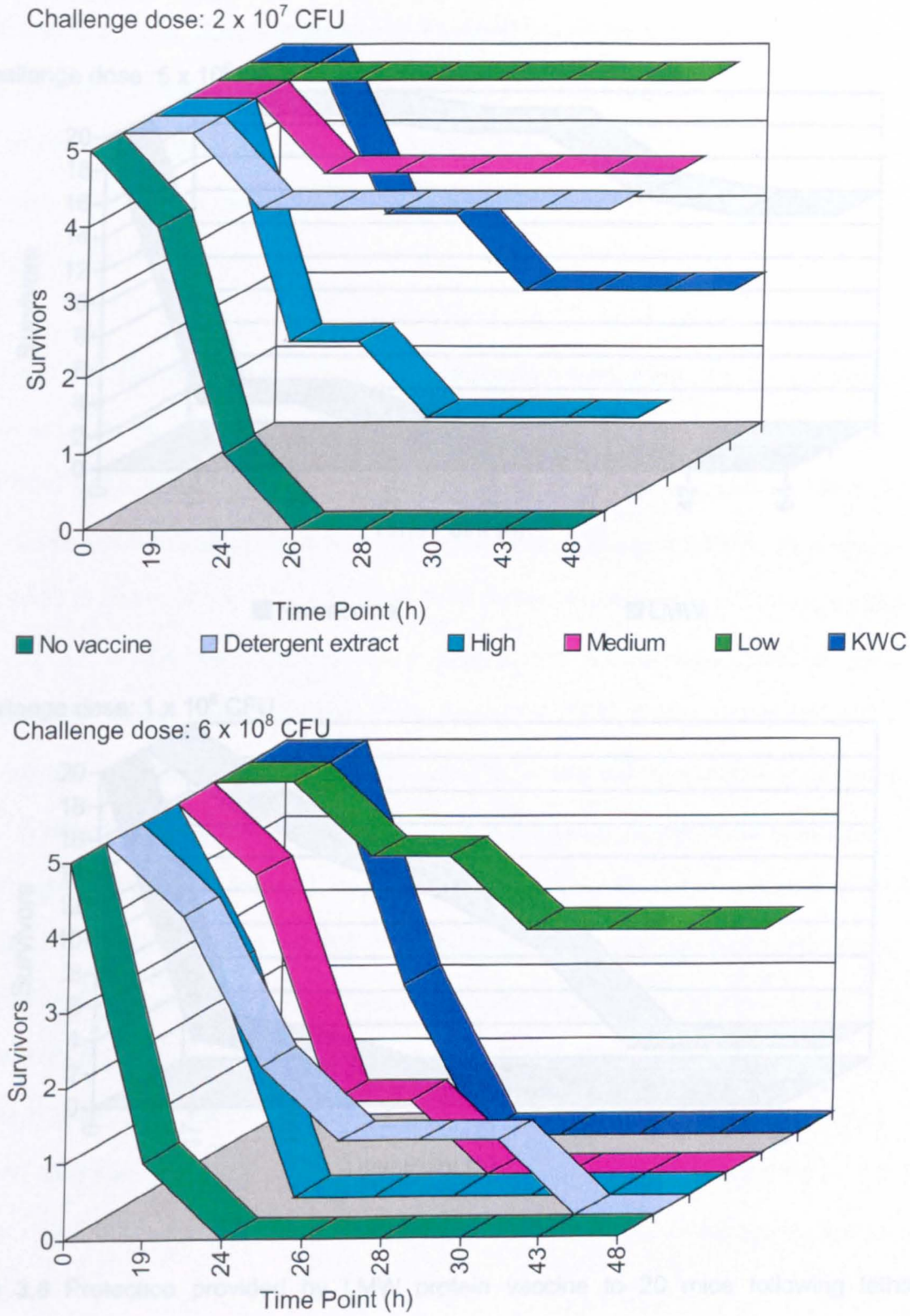

Figure 3.5 Protection provided to groups of five mice by *N. lactamica* vaccines prepared from killed whole cells, detergent extracted proteins or the LMW, MMW or HMW protein pools against meningococcal challenge doses of 2 x 10^7 CFU and 6 x 10^8 CFU. The control group was unvaccinated.

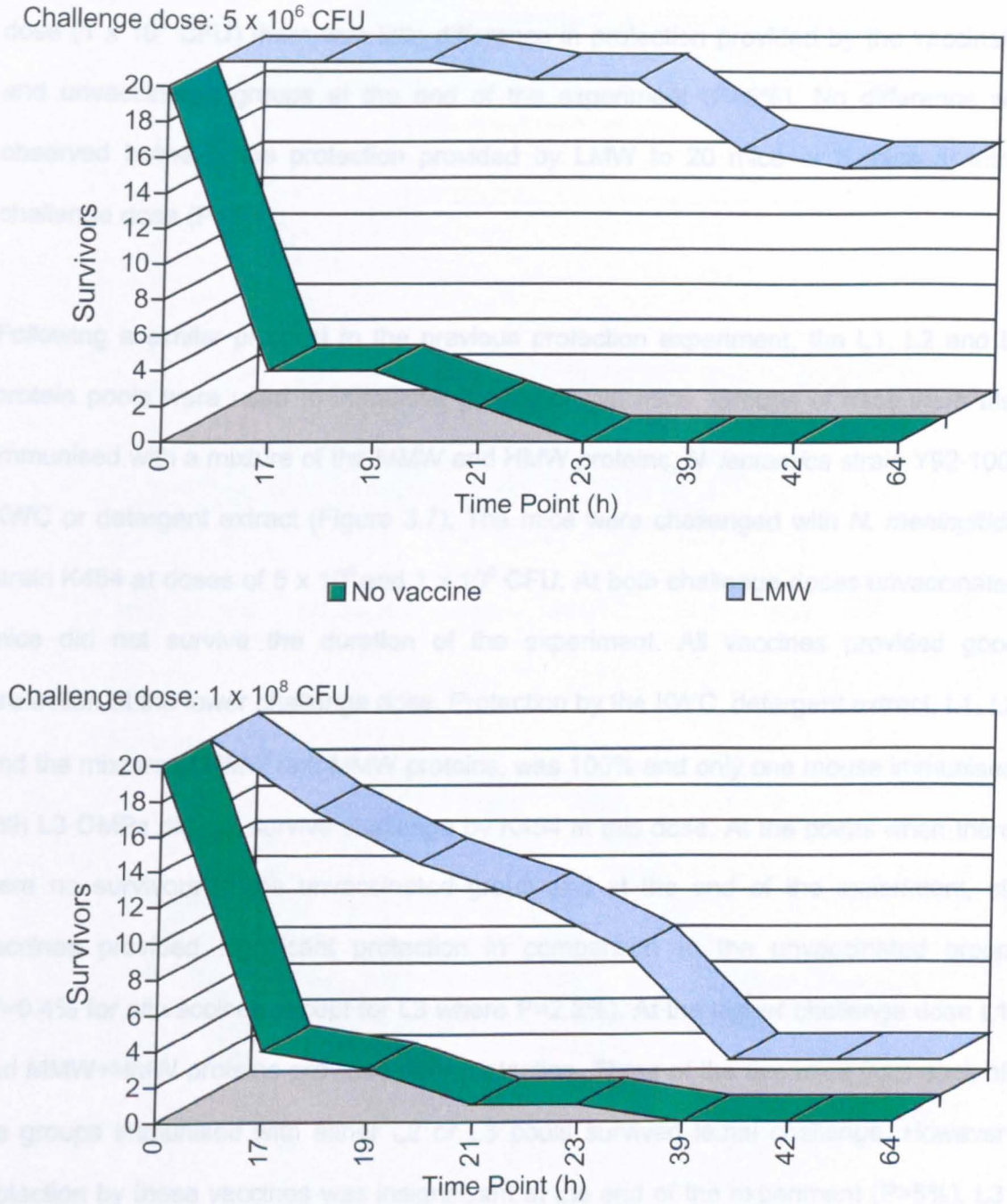

Figure 3.6 Protection provided by LMW protein vaccine to 20 mice following lethal meningococcal challenge at 5 x 10^6 CFU and 1 x 10^8 CFU. The control group was unvaccinated.

experiment. There were no survivors in the unvaccinated group. Protection was highly significant at the low challenge dose at both time points (P<1%). At the higher challenge dose (1 x 10^8 CFU) there was little difference in protection provided by the vaccinated and unvaccinated groups at the end of the experiment (P>5%). No difference was observed between the protection provided by LMW to 20 mice or 5 mice at either challenge dose (P>5%).

Following a similar protocol to the previous protection experiment, the L1, L2 and L3 protein pools were used to immunise groups of five mice. Groups of mice were also immunised with a mixture of the MMW and HMW proteins, *N. lactamica* strain Y92-1009 KWC or detergent extract (Figure 3.7). The mice were challenged with *N. meningitidis* strain K454 at doses of 5 x 10^6 and 1 x 10^8 CFU. At both challenge doses unvaccinated mice did not survive the duration of the experiment. All vaccines provided good protection at the lower challenge dose. Protection by the KWC, detergent extract, L1, L2 and the mixture of HMW and MMW proteins, was 100% and only one mouse immunised with L3 OMPs did not survive challenge by K454 at this dose. At the points when there were no survivors in the unvaccinated group and at the end of the experiment, all vaccines provided significant protection in comparison to the unvaccinated group (P=0.4% for all vaccines except for L3 where P=2.3%). At the higher challenge dose L1 and MMW+HMW proteins provided poor protection. Three of the five mice from each of the groups immunised with either L2 or L3 pools survived lethal challenge. However protection by these vaccines was insignificant at the end of the experiment (P>5%). L3 provided protection at the point when there were no survivors in the unvaccinated group (P=0.4%).

3.2.3. Quantification of Endotoxin in the OMP Pools.

A Limulus Amoebocyte Lysate (LAL) assay was carried out to quantify the amounts of endotoxin in each of the protein pools used in the protection experiments to determine whether LOS had a role to play in the protection observed by the LMW, L2 and L3

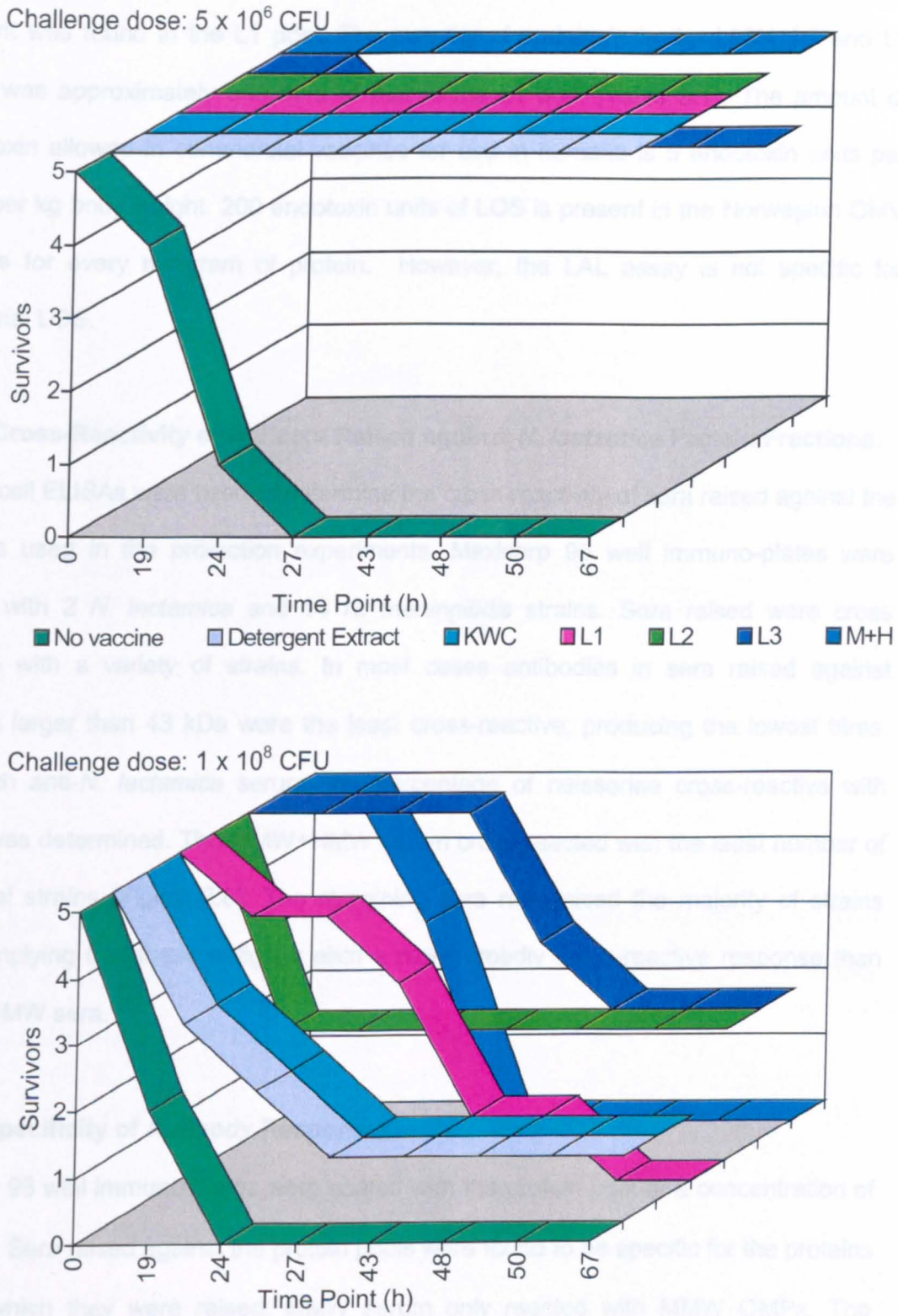

Figure 3.7 Protection provided to groups of five mice by *N. lactamica* vaccines prepared from killed whole cells, detergent extracted proteins or the L1, L2, L3 or a mixture of MMW and HMW protein pools against two lethal meningococcal challenge doses of 5 x 10^6 CFU and 1 x 10^8 CFU. The control group was unvaccinated.

protein pools. Endotoxin was found in all of the protein groups tested but the greatest amount was found in the L1 pool. The quantity of endotoxin in the LMW, L2 and L3 pools was approximately one third of that in the L1 pool (Table 3.1). The amount of endotoxin allowed in commercial vaccines for use in humans is 5 endotoxin units per dose per kg body weight. 200 endotoxin units of LOS is present in the Norwegian OMV vaccine for every milligram of protein. However, the LAL assay is not specific for neisserial LOS.

3.2.4. Cross-Reactivity of Antisera Raised against *N. lactamica* Protein Fractions.

Whole cell ELISAs were used to determine the cross-reactivity of sera raised against the proteins used in the protection experiments. Maxisorp 96 well immuno-plates were coated with 2 *N. lactamica* and 14 *N. meningitidis* strains. Sera raised were cross reactive with a variety of strains. In most cases antibodies in sera raised against proteins larger than 43 kDa were the least cross-reactive, producing the lowest titres. For each anti-*N. lactamica* serum, the percentage of neisseriae cross-reactive with serum was determined. The MMW+HMW serum cross-reacted with the least number of neisserial strains (Figure 3.8). The remaining sera recognised the majority of strains tested implying that these antigens elicit a more broadly cross-reactive response than MMW+HMW sera.

3.2.5. Specificity of Antibody Responses.

Maxisorp 96 well immuno-plates were coated with the protein pool at a concentration of 2 $\mu g\ ml^{-1}$. Sera raised against the protein pools were found to be specific for the proteins against which they were raised. MMW serum only reacted with MMW OMPs. The remaining sera reacted with LMW, MMW and HMW OMPs (Figure 3.9a). However HMW serum was most specific for MMW OMPs. KWC, DE and LMW sera were most reactive with LMW proteins, suggesting that LMW OMPs are highly immunogenic. Cross-reactivity of the MMW+HMW serum was undetectable with L1 and L2 OMPs. L1 sera did not react with L3 and MMW+HMW nor L3 serum with L1 OMPs (Figure 3.9b).

Sample	EU/ml	EU/vaccine	μg/ml
L1	26580	5316	0.53
L2	9149	1830	0.18
L3	9348	1870	0.19
LMW	8515	1703	0.17
MMW	1523	305	0.03
HMW	118	24	0.02
DE	28190	5638	0.56

Table 3.1 Endotoxin titres of *N. lactamica* vaccine formulations determined by LAL assay. EU = endotoxin units. Due to the low molecular weight of LOS the higher molecular weight vaccines (MMW and HMW) consist of the least endotoxin.

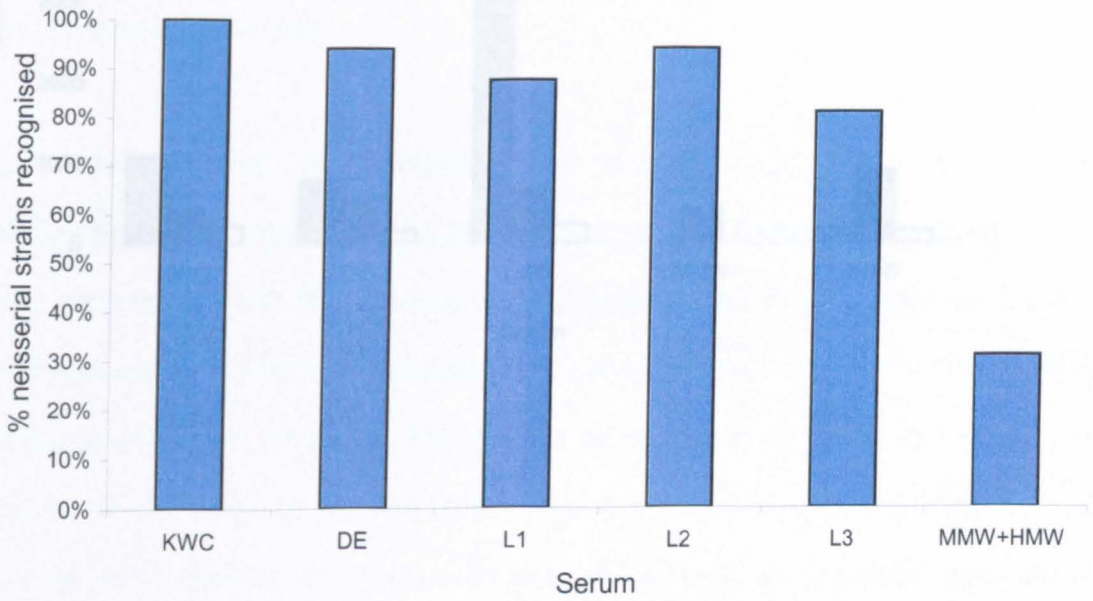

Figure 3.8 Percentage of neisserial strains, including a variety of serogroups, serotypes and serosubtypes, reacting with *N. lactamica* sera determined by whole cell ELISA with *N. lactamica*, strains Y92-1009 and Y92 1011 and *N. meningitidis*, strains K454, GN, BM, L91-113, L2412, L91-543, L93-658, L91-705, JB, LV, L94-4931, AR, B16B6 and H44/76.

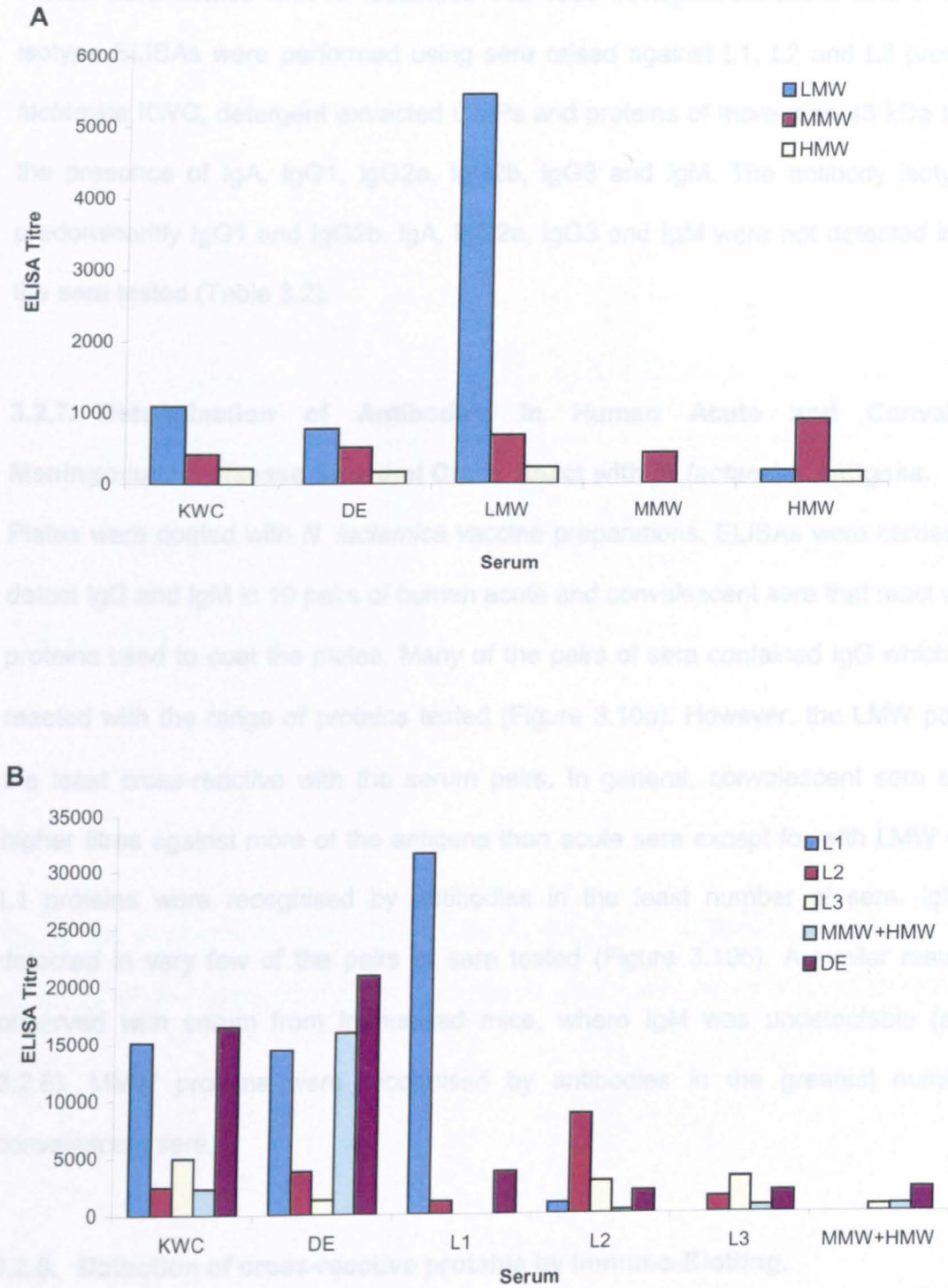

Figure 3.9 Specificity of anti-*N. lactamica* serum towards the homologous proteins. **A** Cross-reaction of KWC, DE, LMW, MMW or HMW sera with LMW, MMW and HMW proteins. **B** Cross-reaction of KWC, DE, L1, L2, L3 or MMW+HMW, sera with L1, L2, L3, MMW+HMW and DE proteins.

3.2.6. Antibody Isotype Responses.

Plates were coated with *N. lactamica* Y92-1009 detergent-extracted OMPs. Antibody isotype ELISAs were performed using sera raised against L1, L2 and L3 proteins, *N. lactamica* KWC, detergent extracted OMPs and proteins of more than 43 kDa to detect the presence of IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM. The antibody isotype was predominantly IgG1 and IgG2b. IgA, IgG2a, IgG3 and IgM were not detected in any of the sera tested (Table 3.2).

3.2.7. Determination of Antibodies in Human Acute and Convalescent Meningococcal disease Sera that Cross-React with *N. lactamica* Antigens.

Plates were coated with *N. lactamica* vaccine preparations. ELISAs were carried out to detect IgG and IgM in 10 pairs of human acute and convalescent sera that react with the proteins used to coat the plates. Many of the pairs of sera contained IgG which cross-reacted with the range of proteins tested (Figure 3.10a). However, the LMW pool was the least cross-reactive with the serum pairs. In general, convalescent sera showed higher titres against more of the antigens than acute sera except for with LMW OMPs. L1 proteins were recognised by antibodies in the least number of sera. IgM was detected in very few of the pairs of sera tested (Figure 3.10b). A similar result was observed with serum from immunised mice, where IgM was undetectable (section 3.2.6). MMW proteins were recognised by antibodies in the greatest number of convalescent sera.

3.2.8. Detection of cross-reactive proteins by Immuno-Blotting.

Elugent extracts from a variety of *N. meningitidis* strains, including those expressing class 2 or class 3 PorB, were separated by SDS-PAGE and transferred to nitro-cellulose. Blots were then incubated with sera raised against *N. lactamica* OMPs to identify the proteins recognised by antibodies (Figure 3.11). L1 serum reacted with fewer proteins than sera raised against other protein groups. This was probably due to this serum containing antibodies directed mainly against LOS. The most obvious bands

Serum	Conjugate					
	αIgA	αIgGM	αIgG1	αIgG2a	αIgG2b	αIgG3
KWC	<100	<100	3382	<100	669	<100
DE	<100	<100	9190	<100	272	<100
L1	<100	<100	2430	<100	<100	<100
L2	<100	<100	2177	<100	<100	<100
L3	<100	<100	679	<100	<100	<100
MMW+HMW	<100	<100	504	<100	<100	<100

Table 3.2 Antibody isotype 50% endpoint titre of *N. lactamica* antiserum. Cross-reactivity of the sera was assessed with *N. lactamica*, strain Y92-1009, detergent extract.

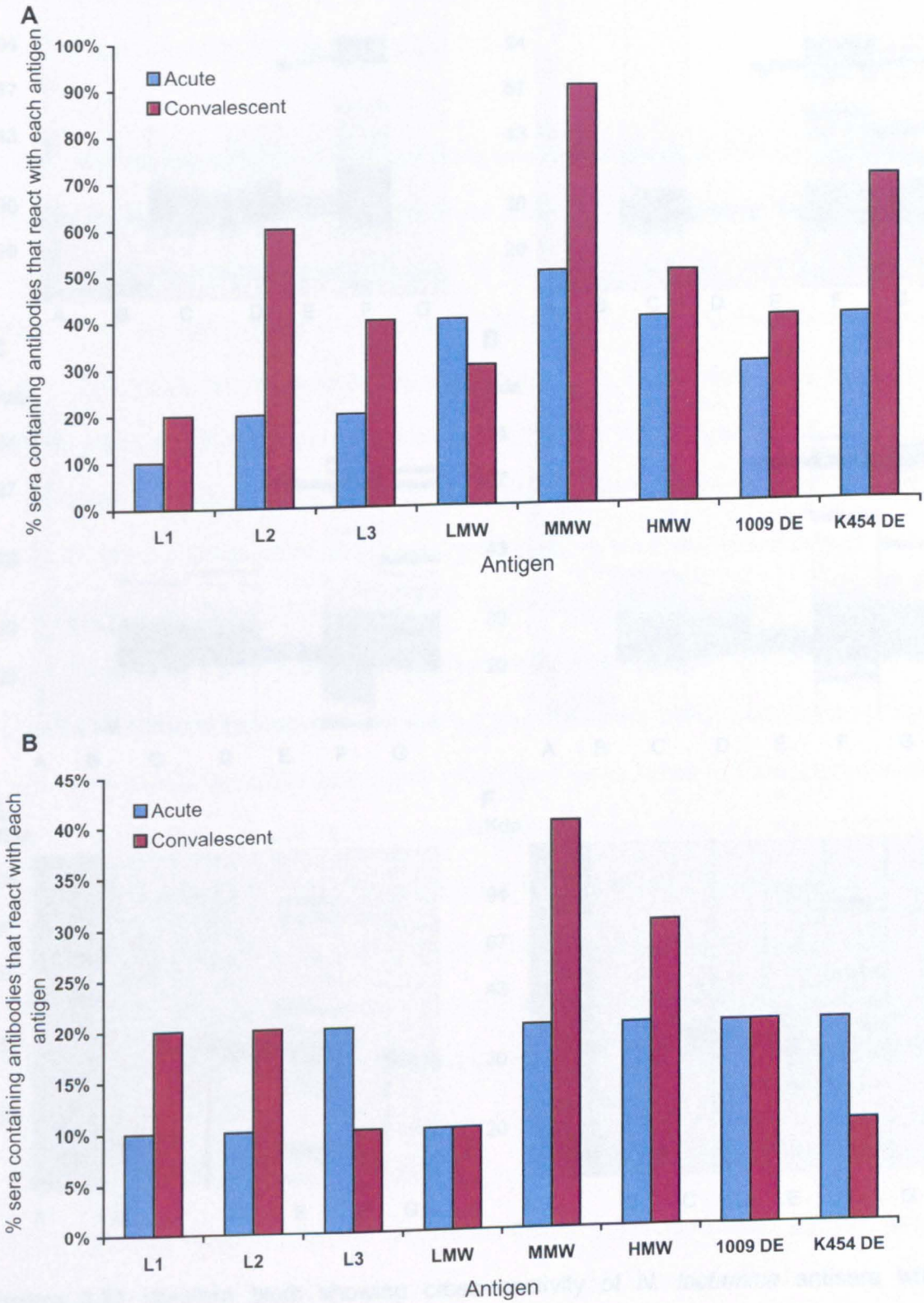

Figure 3.10 Percentage of human meningococcal disease acute and convalescent antiserum IgG (**A**) and IgM (**B**) containing antibodies that react with *N. lactamica* OMPs. Ten serum pairs were tested.

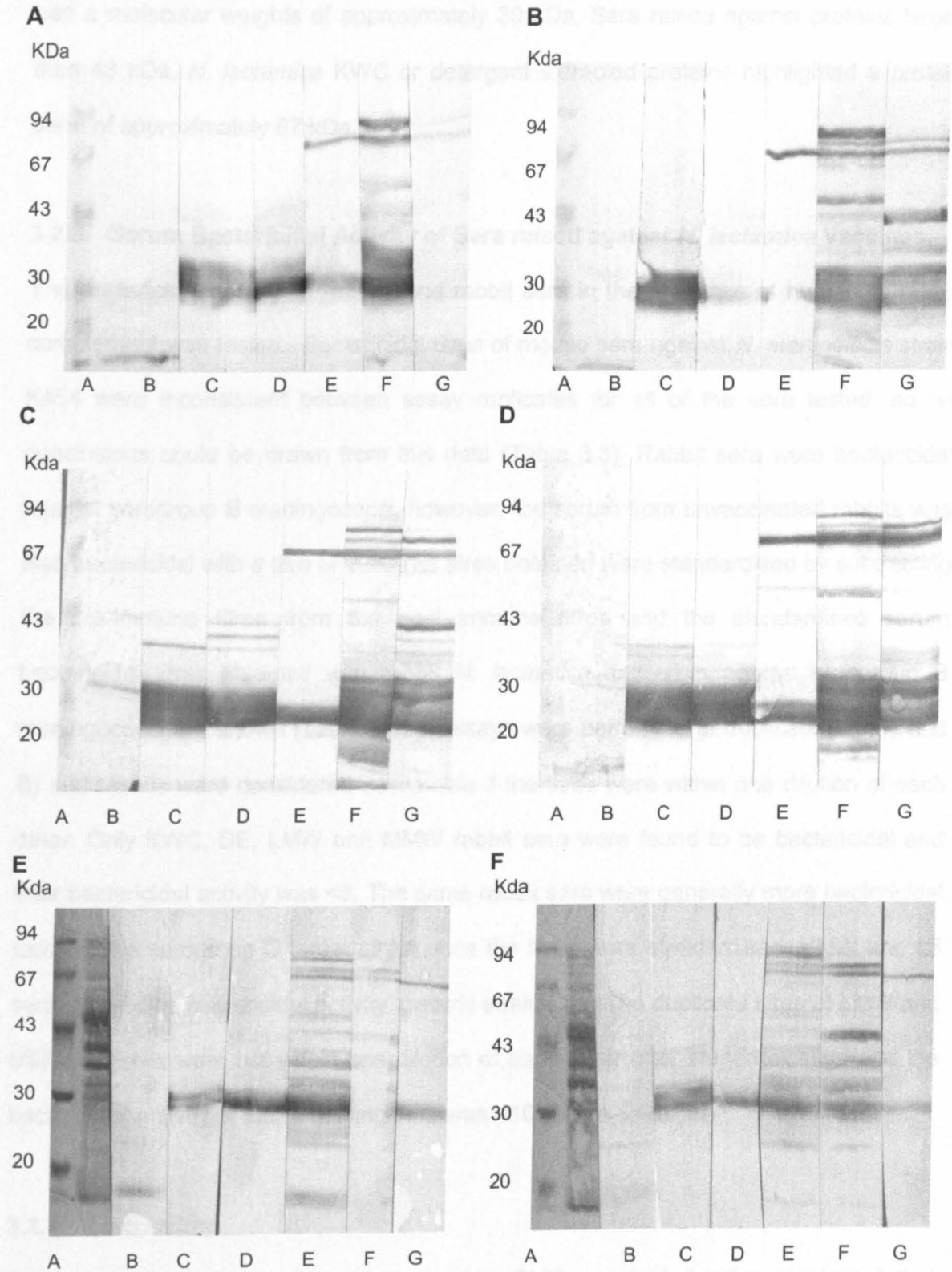

Figure 3.11 Western blots showing cross-reactivity of *N. lactamica* antisera with detergent extracts from 6 meningococcal strains; **A** K454, **B** GN, **C** L91-705, **D** H44/76, **E** M99-242020, **F** M99-242180. The molecular weight markers are shown on lane A. Lanes B-G are probed with L1, L2, L3, KWC, DE and MMW+HMW antisera respectively.

had a molecular weights of approximately 30 kDa. Sera raised against proteins larger than 43 kDa, *N. lactamica* KWC or detergent extracted proteins highlighted a protein band of approximately 67 kDa.

3.2.9. Serum Bactericidal Activity of Sera raised against *N. lactamica* Vaccines.

The bactericidal activity of mouse and rabbit sera in the presence of human or rabbit complement was tested. Bactericidal titres of mouse sera against *N. meningitidis* strain K454 were inconsistent between assay replicates for all of the sera tested, so no conclusions could be drawn from this data (Table 3.3). Rabbit sera were bactericidal against serogroup B meningococci, however, the serum from unvaccinated rabbits was also bactericidal with a titre of 128. The titres obtained were standardised by subtracting the pre-immune titres from the post immune titres and the standardised serum bactericidal titres obtained with rabbit *N. lactamica* antiserum against serogroup B meningococci are shown (Table 3.4a). Assays were performed in duplicate (titre A and B) and results were considered acceptable if the titres were within one dilution of each other. Only KWC, DE, LMW and MMW rabbit sera were found to be bactericidal and their bactericidal activity was <8. The same rabbit sera were generally more bactericidal towards the serogroup C target strain once the titres were standardised. HMW and L3 sera showed no bactericidal activity towards strain C11. The duplicate titres of LMW and MMW antisera were not within one dilution of each other after standardisation and the bactericidal activity of the remaining sera was <16 (Table 3.4b).

3.3. Discussion

This study has shown that *N. lactamica* OMPs protected mice against lethal meningococcal challenge. The immunogenicity of *N. lactamica* OMPs was also assessed. Antibodies were cross-reactive with a variety of meningococcal strains assessed by both ELISA and western blotting. Bactericidal studies with mouse sera were inconclusive but from the results shown in this chapter, *N. lactamica* proteins of <43kDa (LMW) were observed to be important in protection.

Serum	SBA 1	SBA 2	SBA 3
L1	128	4	4
L1	8	8	4
L2	64	4	4
L2	32	32	8
L3	128	8	4
L3	32	4	8
MMW+HMW	32	8	4
MMW+HMW	16	8	4
KWC	8	8	4
KWC	16	8	4
DE	4	8	4
DE	4	8	4

Table 3.3 Mouse Serum Bactericidal Assay Raw Data showing the serum dilution giving >50% killing. The assay was repeated in triplicate with K454 as the target strain and a human complement source. Bactericidal titres of the duplicates of both L1 and L3 anitsera were not within one dilution of each other initially (SBA1). On repetition of the assay, SBA2 and SBA3, the titres were much lower than initially observed rendering all results inconclusive.

A

Immunogen	Titre A	Titre B
KWC	8	4
DE	8	8
LMW	4	8
MMW	4	<4
HMW	<4	<4
L1	<4	<4
L2	<4	<4
L3	<4	<4

B

Immunogen	Titre A	Titre B
KWC	16	8
DE	8	8
LMW	32	8
MMW	16	4
HMW	<4	<4
L1	8	4
L2	4	<4
L3	<4	<4

Table 3.4 Standardised serum bactericidal antibody titres obtained with rabbit *N. lactamica* antiserum against **A,** serogroup B, and **B**, serogroup C target strains.

3.3.1. Protection by *N. lactamica* OMPs.

It has been suggested that carriage of *N. lactamica* by young children may be involved in natural protection against meningococcal disease due to the high carriage rates of *N. lactamica* in children under the age of five years in comparison to *N. meningitidis* carriage (Gold *et al.*, 1978). Although the symptoms of meningitis are not observed in the mouse i.p. protection model, the model is useful in assessing the protective efficacy of vaccines as infection by *N. meningitidis*, augmented by hTf, is lethal to mice. Protection experiments involving sub-cutaneous vaccination of mice with *N. lactamica* KWC have demonstrated a protective effect.

Attention has been focussed on OMPs of *N. meningitidis* by a number of groups for the production of a vaccine protecting against meningococcal disease (Morley *et al.*, 2002). However, heterogeneity of the major OMPs creates problems for a vaccine of this type, as a successful vaccine must contain the OMPs expressed by a variety of strains to provide broad protection. Poolman *et al.*, (1991) reviewed the work of a number of authors who have found that OMPs which produced a cross-reactive antibody response to a variety *N. meningitidis* strains were not bactericidal, but OMPs which were variable among meningocccal serotypes and serosubtypes, such as class 1, 2 and 3 proteins, generated antibodies which were bactericidal. Despite their variablilty, they investigated the immunogenicity of a multivalent PorA OMV vaccine as a vaccine against serogroup B meningococci. Although this induced bactericidal antibodies, the bactericidal activity was directed only towards the homologous strains (Cartwright *et al.*, 1999). An ideal meningococcal vaccine would provide protection that is serogroup, serotype and serosubtype independent. Due to the cross-reactive immunity provided by *N. lactamica* with a variety of meningococcal strains described in this chapter, *N. lactamica* OMPs may provide protection that is not strain specific.

OMPs isolated from *N. lactamica* were shown to provide similar levels of protection to mice as *N. lactamica* killed whole cells. To identify the OMPs that provided protection,

the detergent extracted OMPs were separated by preparative electrophoresis and pooled into three groups of proteins. Protection was found to be greatest for LMW proteins (<43 kDa). Of the protein pools, HMW proteins (proteins of more than 65 kDa) provided the least protection. However, Sierra *et al.*, (1990) enriched an OMV vaccine with native high molecular weight proteins. These were shown to have the capacity to induce long lasting bactericidal antibodies. This vaccine was effective in preventing disease in children over 4 years of age in clinical trials. Wedege *et al.*, (1986) showed that sera from humans vaccinated with *N. meningitidis* OMVs contained antibodies that reacted with *N. meningitidis* OMPs of 140, 86, 65, 43, 30 and 22 kDa. However, patterns of reactivity varied between individuals. This study has shown that *N. lactamica* LMW proteins provided protection to mice suggensting that the 43, 30 and 22 kDa proteins may be important in mounting a protective immune response against meningococcal disease. Wedege *et al.* (1986) identified the 43 kDa protein as the class 1 OMP (PorA) and the 30 kDa protein as the class 5 protein (Opa).

LMW proteins were further separated into protein pools of less than 25 kDa (L1), 25-35 kDa (L2) and 35-43 kDa (L3). Good protective efficacy was provided by L2 and L3 proteins at the 1 x 10^8 CFU challenge dose, better even than KWC and *N. lactamica* detergent extract. Although all mice were vaccinated with 10μg of protein, the concentration of the 25-35 kDa and 35-43 kDa proteins in the L2 and L3 vaccines, respectively, is greater than the concentration of these proteins in the killed whole cell and detergent extract vaccines. This may account for their improved protection.

3.3.2. Involvement of LOS in Protection.

Antibodies directed towards LOS are raised during meningococcal infection and LOS is important in the pathogenesis of meningococcal disease. Although a number of authors have suggested and tested the use of LOS in vaccine formulations, these have not been successful due to LOS heterogeneity (Verheul *et al.*, 1993, Poolman *et al.*, 1991). Vaccines for human use are required to contain a limited amount of LOS i.e. less than 5

endotoxin units per dose per kg body weight. Measurement of circulating endotoxin in patients with systemic meningococcal infection has shown that levels of endotoxin exceed 700 ng per litre (Schlichting *et al.*, 1993). By comparison endotoxin is undetectable in the serum of patients with meningitis and the uninfected.

The LAL assay was used to determine the role of LOS in the protection provided by LMW OMPs (section 3.2.3). The quantity of LOS in the LMW protein pool was between five and six times that found in the MMW and HMW protein pools. However, the quantity of LOS in the L1 protein pool was approximately three times greater than in the L2 and L3 proteins. This group was less protective than L2 and L3, suggesting that LOS did not play a major role in the observed protection. However, these results cannot be relied upon as the LAL assay does not specifically detect LOS, but endotoxin as a whole. Generally, LAL assay results are difficult to interpret due to the presence of non-specific activators and inhibitors of LOS. Using the LAL assay, Schlichting *et al.*, (1993) had trouble quantifying the LOS in different culture filtrates due to the variable Limulus-activating potential of LOS from different meningococcal strains. Furthermore, the vaccine formulations may have been contaminated with endotoxin at some point during preparation or quantification, adding to the difficulties with this method for LOS quantification and comparison of the different vaccines. Tsai *et al.*, (1989) used the LAL assay to measure LOS in vaccine preparations. They found that this assay was inaccurate for the measurement of LOS when it was in a mixture with proteins. Protein bound LOS was 20 to 40 fold less reactive than purified LOS by LAL assay. They also found that in a mixture with proteins, LOS was not as toxic to rabbits as purified LOS suggesting that protein vaccine formulations could be used even when the quantity of LOS is higher than would normally be allowable for purified LOS in human vaccines.

3.3.3. Cross-Reactivity of *N. lactamica* Antiserum with *N. meningitidis.*

ELISA and immuno-blotting techniques showed that all sera raised against *N. lactamica* in this study were cross-reactive for a wide range of meningococcal strains including

serogroups A, B and C. Sera raised against MMW+HMW proteins produced lowest titres towards *N. meningitidis* whole cells and were also the least cross-reactive, reacting with only 31% of strains tested. L2 serum cross-reacted with the same percentage of strains as the detergent extract serum but the cross-reaction of L1 and L3 sera with the panel of neisserial strains was also good (>80% of strains).

Immuno-blots also showed that *N. lactamica* antisera are cross-reactive with detergent extracts from a variety of *N. meningitidis* strains (section 2.4). Both PorB class 2 or 3 positive meningococcal strains were tested and the patterns of cross-reaction were similar for all strains, indicating that sera raised against all *N. lactamica* vaccines tested did not cross-react in a serotype-specific manner. A meningococcal protein of approximately 30 kDa was recognised by all sera tested and sera raised against *N. lactamica* OMPs of more than 43 kDa, killed whole cells or detergent extracted proteins cross-reacted with a protein of approximately 70 kDa. Two human convalescent immune sera from patients recovering from meningococcal disease and two mouse sera raised against *N. lactamica* strain PO33 and *N. meningitidis* strain GLD have recently been shown by Troncoso *et al.*, (2000) to recognise *N. meningitidis* proteins of 65, 55 and 32 kDa. The 32 kDa protein was not regulated by iron levels and it was suggested that it could be a class 4 OMP. The cross-reactive protein of approximately 30 kDa could also be either PorB class 2 or 3 (37-42 kDa), Opa (26-30 kDa) or Opc (25 kDa). However, Kim *et al.*, (1989) suggest that *N. lactamica* do not possess OMPs that are antigenically similar to meningococcal class 2 or 3 proteins. The 70k Da protein could be FetA (70 kDa) and probing *N. meningitidis* strains grown under iron limited and iron replete conditions may confirm this.

Now that the genome sequence of *N. meningitidis* strain MC58 has been completed, it would be of interest to determine the proteins of *N. lactamica* that are cross reactive with immunogenic recombinant proteins described by Pizza *et al* 2000.

3.3.4. Isotype Response of *N. lactamica* Cross-Reacting Antiserum.

Four subclasses of IgG are found in mouse serum; IgG1, IgG2a, IgG2b and IgG3. IgG1 was found to be the predominant antibody isotype of sera raised against *N. lactamica* OMP fractions, KWC or detergent extract. Low IgG2b antibody titres were detected in sera raised against killed whole cells or Elugent extract. IgM was not detected in the sera but this was to be expected as the assay was detecting secondary antibody responses to the vaccinations.

Previous investigations of predominant isotypes of the antibodies evoked by LOS conjugation to TT or OMP showed that the main antibody response was by IgG1 (Verheul *et al.*, 1993). This is consistent with results found in this study (section 3.2.6); IgG1 was also the predominant isotype in sera reacting with the meningococcal porins (Lehmann *et al.*, 1999, Guttormsen *et al.*, 1993). In humans IgG1 and IgG3 are the most effective of the IgG isotypes at triggering complement activation and binding to the Fc receptors. However, of the antibody subclasses present in mice, IgM, IgG2a, IgG2b and IgG3 are complement activators. IgG1 displays no activity and is even capable of inhibiting the lytic activity of complement activating subclasses (Ey *et al.*, 1980).

3.3.5. Human Antibody Responses.

After vaccination, IgM and subsequently IgG antibodies are induced in humans. Serum IgA may also be formed. The route of administration and site of immunisation affect the subclass and isotype response of the antibodies. For example oral administration of a vaccine will produce a localised mucosal immune response. In man, protein antigens induce IgG1 antibodies and carbohydrates induce IgG2 (Hammarstrom *et al.*, 1986). This study focused only on the antibody subclass response in humans and not the IgG isotype responses.

Detection of IgG in human acute and convalescent sera cross-reactive with *N. lactamica* did not follow a particular pattern (section 3.2.7). IgG in sera was not directed

specifically towards a particular group of proteins. Nine of the ten convalescent sera tested contained antibodies that cross-reacted with MMW proteins but only three of the convalescent sera possessed antibodies that were cross-reactive for LMW proteins. Although LMW proteins were found to be the most protective group of *N. lactamica* OMPs, similar proteins of *N. meningitidis* may not be the most immunogenic proteins in humans. One serum pair reacted with all proteins tested with highest titres detected for cross-reaction with MMW and HMW proteins and *N. lactamica* detergent extract. The same serum was also found to consist of IgM cross-reactive for all protein groups tested, but highest titres were for the cross-reaction with MMW proteins. Paired sera from another patient did not recognise any of the protein groups. IgM that was cross-reactive for *N. lactamica* OMPs was detected in very few of the human sera tested. Results involving cross-reactivity of human disease sera with *N. lactamica* OMPs are inconclusive. However, cross-reacting antibodies against *N. meningitidis* outer membrane complexes have been demonstrated in convalescent sera from patients with serogroup B disease (Guttormsen *et al.*, 1992).

Mandrell *et al.*, (1989) quantified the OMP specific antibodies in human convalescent and post vaccination sera. As shown in section 3.2.7 they also found that there was considerable heterogeneity among individual human antibody responses. They assessed the reactivity of human sera with 3 different OMPs; class 1, 2 and 5. Some individuals had a high response to one protein but none to the others and other individuals had no measurable response to any of the proteins. They suggested that a difference in response may be due to the presence or absence of pre-existing antibody or to variable expression of antigens in meningococcal disease patients and vaccinees. Heterogeneity among antibody responses to *N. meningitidis* OMPs has also been shown by Wedege *et al.*, (1986) by Western blotting. IgG antibodies in some sera recognised only one OMP whereas other sera detected up to six OMPs. Mandrell *et al.*, (1989) also suggested the following reasons for differences in antibody responses of sera from different individuals; primed immune system, persistence of infection or

vaccine antigen, different proportions of inhibiting and non-inhibiting antibody and antibody affinity.

Human antibody ELISAs in this study have shown that circulating IgM of human sera are far less than IgG responses to the *N. lactamica* OMPs. Wedege *et al.*, (1986) found the same result by western blotting with far fewer individuals mounting an IgM response to the OMPs than mounted an IgG response.

3.3.6. Serum Bactericidal Activity of *N. lactamica* Antiserum.

Natural protection in young children by *N. lactamica* carriage is suggested to result in the production of bactericidal antibodies. Gold *et al.*, (1978) found that forty percent of carriers of *N. lactamica* developed bactericidal antibody titres directed towards *N. meningitidis* of serogroups A, B and C. Bactericidal activity is associated with protection against meningococcal disease (Goldscheider *et al.*, 1969). Prior to disease outbreaks in, for example, students (Jones *et al.*, 2000), the bactericidal titre of the population is low and susceptibility is high. In this study, the bactericidal activity of mouse and rabbit serum against serogroup B and C meningococci with human and rabbit complement, respectively, was assessed. The sera tested were raised against *N. lactamica* pooled proteins.

The 'gold standard' correlate of protection against serogroup B and C meningococcal disease is a serum bactericidal titre of 4 or more with human complement (Goldschneider *et al.*,1969, Borrow *et al.*, 2001). However good sources of human complement that lack antibodies against *N. meningitidis* are not readily available so rabbit complement has been assessed (Borrow *et al.*, 2001). Using rabbit complement, a titre of 8 was suggested as a correlate of protection. Titres of less than 8 with rabbit complement predict susceptibility to meningococcal disease and titres of more than 128 are highly predictive of protection. Similarly, Santos *et al.*, (2001) suggested that a human bactericidal antibody titre of more than 128 is required when using rabbit

complement to be sufficiently confident of predicted protection. Use of rabbit complement elevates bactericidal antibody titres to serogroup C meningococci in comparison with human complement. They recommend the use of human complement for assays because the use of a threshold titre of 128 with rabbit complement is less sensitive. Milagres *et al.*, (2000) assessed the bactericidal activity of human convalescent sera against serogroup B meningococci. They found that titres of more than 2 indicated a specific immune response.

Serum bactericidal assays involving sera raised in mice against *N. lactamica* OMPs provided disappointing results. Sera were found to have variable and very low levels of bactericidal antibodies cross-reactive with *N. meningitidis*. Protection experiments carried out in this study consisted of only 2 booster vaccinations after primary immunisation and Cartwright *et al.*, (1999) found that bactericidal titres to a novel hexavalent PorA vaccine after two booster vaccinations post primary immunisation were unimpressive. A third booster vaccination after primary immunisation was found to much improve bactericidal titres. Bactericidal titres for mouse sera raised against *N. lactamica* OMP sub-unit vaccines may be increased with a third boost. West *et al.*, (2001) showed that mouse serum raised against recombinant Tbp A was not bactericidal although protection was conferred by this protein. They suggested that the serum bactericidal assay may not, therefore, be a good predictor of protection in humans either. Similary, Perkins *et al.*, (1998), suggest that serum bactericidal titres may be an insensitive correlate for protective efficacy for some outer membrane protein vaccines. When a serum bactericidal titre is present it is likely to correlate to protective efficacy but lack of bactericidal titre does not necessarily imply that a vaccine is not protective. They suggest that antibodies that are not bactericidal may be protective through other mechanisms such as opsonophagocytosis. An alternative reason for the low bactericidal titres found may be the lack of a PorA protein expressed by *N. lactamica* (Kim *et al.*, 1989). Bactericidal antibodies are often directed towards PorA proteins of *N. meningitidis*. Removal of PorA from a vaccine shows a strong reduction in bactericidal

antibody titres but removal of PorB has little effect (Van der Ley, 1992, Saukkonen, 1989).

The rabbit sera tested were bactericidal; however the pre-immune serum was also bactericidal. Against serogroup C the sera had a titre of between 4 and 8 and against serogroup B meningococci titres of 128 were observed. In both cases, the bactericidal titres could not be used to predict protection by the vaccines. West *et al.*, (2001) also showed that when compared to mouse sera, rabbit sera were bactericidal, but similarly serum from unimmunised rabbits was also bactericidal.

Granoff *et al.*, (1998) designed an ELISA that correlated to human bactericidal antibody responses. It detected high avidity antibodies by addition of ammonium thiocyanate to the blocking buffer to selectively remove low avidity antibodies. Aase *et al.*, (1995) suggested that there was a weak correlation between serum IgG levels and serum bactericidal activity but that there was good correlation between opsonic activity and serum IgG levels. However, the correlation between bactericidal activity and opsonic activity was weak and they recommend the use of both bactericidal assays and opsonic assays in the assessment of vaccines. It would, therefore be of interest to determine whether the protective antibodies have opsonic activity and whether this can be used to explain the protection observed by the *N. lactamica* proteins.

3.4. Conclusions.

Protection by *N. lactamica* OMPs is not serogroup, serotype or serosubtype specific, suggesting that *N. lactamica* would provide a suitable target for vaccine development against meningococcal disease. Proteins of between 25 and 43 kDa provided the greatest protection to mice, but the protection observed may not be due to bactericidal activity of the antibodies raised. Thus, bactericidal antibodies may not be the best correlate of protection in mice and testing sera for opsonophagocytic or other funtional antibody activity may provide answers as to how LMW *N. lactamica* vaccines protect.

LOS does not appear to be involved in the protection observed by the LMW group, as the quantity of endotoxin was greater in a less protective vaccine formulation. However, the accurate determination of LOS is difficult and thus the results are not conclusive.

Chapter 4

Surface Enhanced Laser Desorption Ionisation to Identify *Neisseria meningitidis* OMPs Cross-Reactive with *Neisseria lactamica* Antiserum

4.1. Introduction.

In chapter 3, immunisation with *N. lactamica* OMPs and derived fractions, was shown to protect mice against lethal meningococcal challenge. *N. lactamica* antiserum was also cross-reactive with detergent extracts from a variety of meningococcal strains suggesting that *N. lactamica* antiserum protects by interaction with specific meningococcal OMPs and that *N. lactamica* OMPs are homologous to meningococcal OMPs. However, the *N. meningitidis* OMPs recognised by *N. lactamica* antiserum are unknown. In this chapter, Surface Enhanced Laser Desorption Ionisation (SELDI) was used to identify *N. meningitidis* OMPs that cross-react with *N. lactamica* antiserum. The serum was bound to tosyl-activated magnetic beads and these were used as an affinity capture surface. Pools of *N. meningitidis* proteins separated by preparative electrophoresis were incubated with the beads, which were then placed directly onto a hydrophobic ProteinChip surface. The proteins bound by the fixed antibody were then desorbed and their molecular mass determined by the SELDI time-of-flight instrument. Using this method the molecular weights of proteins cross-reacting with the serum could be identified. The ultimate aim of this work was to identify the *N. meningitidis* proteins which the protective anti-*N. lactamica* antibodies are directed against.

4.1.1. Mass Spectrometry.

Mass spectrometry (MS) can be applied to all elements and molecules with a molecular mass greater than 15 Da. The basis of MS is the measurement of mass. It requires only very small amounts of sample (analyte) due to the high sensitivity of the technique and several substances can be determined at one time The sample can be in gaseous, liquid or solid form.

The masses of ionised molecules are measured using a mass spectrometer, which can separate charged atoms or molecules according to their mass to charge ratio (m/z). If the ions measured have one charge (i.e. z=1), m/z is equivalent to the molecular mass (www.asms.org, www.bmss.org.uk). The sample is converted to the gaseous phase

either before or during the ionisation process. Once the sample has been ionised, the ions are propelled through an analyser. Analysers are either field instruments, e.g. magnetic sector, quadrupole mass filters, ion traps, or time of flight (ToF) instruments. ToF instruments separate ions by their different flight times over a known distance. Ions are accelerated and directed into a flight tube. Ions with a like charge have equal kinetic energy (kinetic energy is equal to $1/2mv^2$, where v is the velocity of the ion). Therefore, the lower the ion's mass, the greater its velocity and the shorter its time of flight. So like charged ions will have differing times of flight depending on their mass.

A number of techniques have been used for ionisation of the sample, including electron ionisation (EI), fast atom bombardment (FAB), matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). EI involves bombarding the gaseous sample with a beam of electron generating ions. For FAB ionisation the sample is dissolved in a liquid matrix. The function of the matrix is to mediate the transfer of energy from the laser to the sample. The liquid surface is bombarded with a beam of high kinetic energy atoms or ions. ESI involves dissolving the sample in a mixture of water and either acetonitrile or methanol which is then pumped through a fine stainless steel capillary and a high electric potential creates an electrostatic spray of multiply charged droplets. MALDI uses a crystalline matrix and a beam of photons to ionise the sample. Like FAB the sample is dissolved in a matrix such as 2,5-dihydrobenzoic acid or sinapinic acid and allowed to crystallise on a stainless steel target. The surface is then bombarded with a pulsed laser beam and the sample ionised. Ion generation by MALDI is not clearly understood. It is thought that the matrix absorbs most of the photon energy and converts the mixture into vapour. Ions form in the vapour by either proton transfer from the matrix to the analyte forming positive ions or from the analyte to the matrix forming negative ions. Due to the heterogenous mixture of the analyte and matrix best ion formation occurs at particular spots in the preparation (Baar, 2000). The most effective matricies for protein ionisation are α-cyano-4-hydroxycinnamic acid or sinapinic acid.

MALDI is successfully used to generate ions from proteins of over 100 kDa and it has been reported that proteins of up to 500 kDa in *E. coli* lysates have also been detected by MALDI (Chong *et al.*, 1997). MALDI and ESI are now the most commonly used techniques for analyte ionisation.

For MALDI good sample preparation is required to provide the best results. The matrix and analyte may separate upon solvent evaporation. Homogeneity of the sample is critical and mostly depends on the properties of the matrix and the sample. Additives such as trifluoroacetic acid and ethanol help maintain a homogeneous mixture (Hillenkamp *et al.*, 1990). Also matrix ions are often formed and these may covalently bond or non-covalently complex with the analyte. These are known as matrix adducts and they deteriorate the resolution of the mass by broadening the signal of interest (Baar, 2000).

4.1.2. Surface Enhanced Laser-Desorption Ionisation.

In 1993 Hutchens and Yip developed a new form of MALDI. Although essentially the same as MALDI, they redeveloped the sample application surface and called the new technique surface enhanced laser-desorption ionisation (SELDI). Two forms of SELDI were described; surface enhanced neat desorption (SEND) and surface enhanced affinity capture (SEAC). SEND surfaces contained the energy absorbing material so that application of matrix is not required after sample application. For SEAC either the sample application surfaces (probe surface) were used as affinity capture platforms or analyte capture was indirect and performed on beads which were then applied to the probe surface and analysed (Hutchens and Yip, 1993). For both of these techniques matrix was applied as for conventional MALDI. Using SEND peaks on the mass spectrum were symmetrical and there was no evidence of matrix adducts. SEAC also produced good quality results. Using beads they found that the quality of the signal was not compromised by presentation of the analyte on a surface that was not flush with the probe surface. They suggested SEAC as a method for the selective capture of specific

analytes by altering the affinity capture reagents as required. Merchant *et al.*, (2000) reviewed SELDI and suggested that SEAC was the SELDI application showing most promise as the probe surface has a role in sample extraction, presentation and amplification. Molecules bind to the surfaces via hydrophobic, electrostatic covalent bonding and other interactions. Sequential washing steps with the appropriate buffer can isolate molecules with a strong affinity for the chemically active surface. Compounds with shared chemical properties are retained.

Ciphergen (USA) have designed an assortment of chemically-defined surfaces. These are in the form of chips containing 8 or 24 spots with affinity capture surfaces and are called ProteinChip Arrays (www.ciphergen.com). ProteinChip Arrays contain chemically (cationic, anionic, hydrophobic or hydrophilic) or biochemically (antibody, receptor or DNA) defined surfaces. Samples are specifically captured by the surface and can be analysed directly to determine their mass or enzymically digested on the chip to determine their peptide composition. Once the sample has been purified on the chip it is analysed by ToF (Figure 4.1). A matrix is applied and promotes laser based desorption ionisation. Detected analyte is displayed as for MS.

SELDI has been used by Hutchens' group to detect and characterise lactoferricin and lactoferrin (Kuwata *et al.*, 1998a, 1998b, 1998c). Lactoferricin was initially captured specifically using molecules with terminal n-butyl groups. Lactoferricin and lactoferrin fragments in human gastric contents were quantified.

4.1.3. Beads as an Affinity Capture Surface.

Magnetic beads can be used for purifications linked to ToF. Beads are particularly important because minute quantities of proteins can be characterised and the surface area for analyte binding is increased intensifying the signal. Beads can be placed directly on to the probe or ProteinChip and the laser causes the analyte to dissociate from the beads. Although Hutchens and Yip found that there were no problems with the

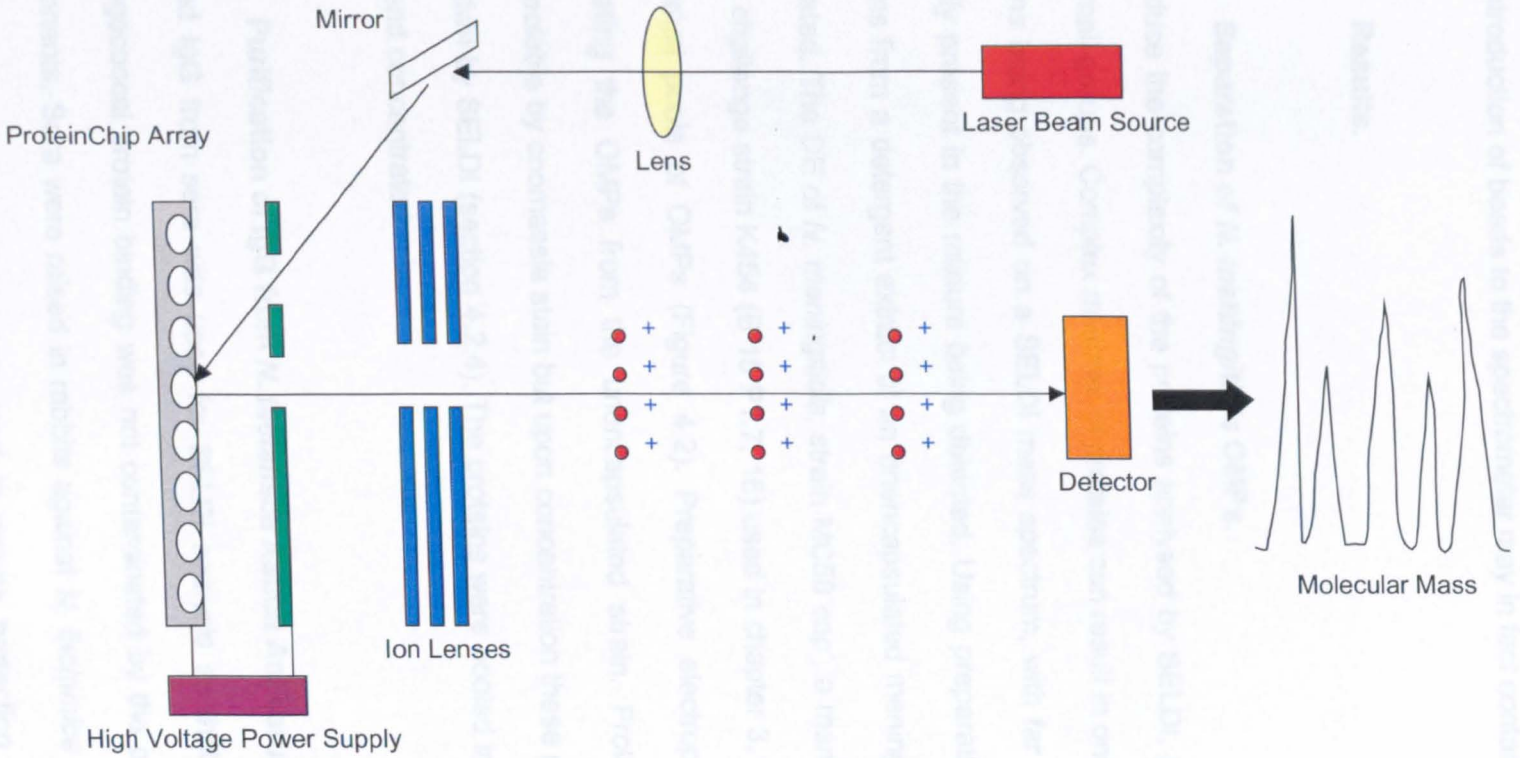

Figure 4.1 Schematic showing the processes involved in SELDI. After inserting the ProteinChip Array into the ProteinChip Reader, a laser beam is fired at the sample, causing the proteins in the matrix to desorb and ionise. Released ions are accelerated in an electrical field causing them to be propelled through a vacuum tube, towards the ion detector. The ionised proteins are detected and an accurate mass is determined based on the ToF (Based on Schematic of ProteinChip Reader, www.ciphergen.com).

resolution of the signal associated with using beads (Hutchens and Yip, 1993) Merchant *et al.*, (2000) suggest that particulates on the probe surface compromise resolution and mass accuracy. Solvents or acid can be used to elute bound sample from the beads immediately prior to analysis. This may enhance the signal produced. They also suggest that introduction of beads to the spectrometer may in fact contaminate the instrument.

4.2. Results.

4.2.1. Separation of *N. meningitidis* OMPs.

To reduce the complexity of the proteins analysed by SELDI, proteins were separated into small groups. Complex mixtures of proteins can result in only the most concentrated proteins being observed on a SELDI mass spectrum, with far fewer proteins than are actually present in the mixture being detected. Using preparative electrophoresis, only proteins from a detergent extract of an unencapsulated meningococcal strain could be separated. The DE of *N. meningitidis*, strain MC58 cap$^-$, a meningococcal strain similar to the challenge strain K454 (B 15 P1.7, 16) used in chapter 3, was fractionated by size into small pools of OMPs (Figure 4.2). Preparative electrophoresis worked well in separating the OMPs from the unencapsulated strain. Proteins of >45 kDa were undetectable by coomassie stain but upon concentration these proteins were detectable as shown by SELDI (section 4.2.4). The proteins were pooled into five groups based on size, and concentrated.

4.2.2. Purification of IgG from *N. lactamica* Rabbit Antiserum.

Purified IgG from sera was used for SELDI analysis so that the mass spectrum of meningococcal protein binding was not contaminated by the presence of other serum components. Sera were raised in rabbits against *N. lactamica* LMW, MMW, HMW, L2 and L3 protein pools, the preps used in mouse protection studies (chapter 3) as described in section 2.7.5. IgG was purified from these sera using a 5ml protein G column. IgG was eluted from the column with 0.1M glycine, pH2. Purified IgG was

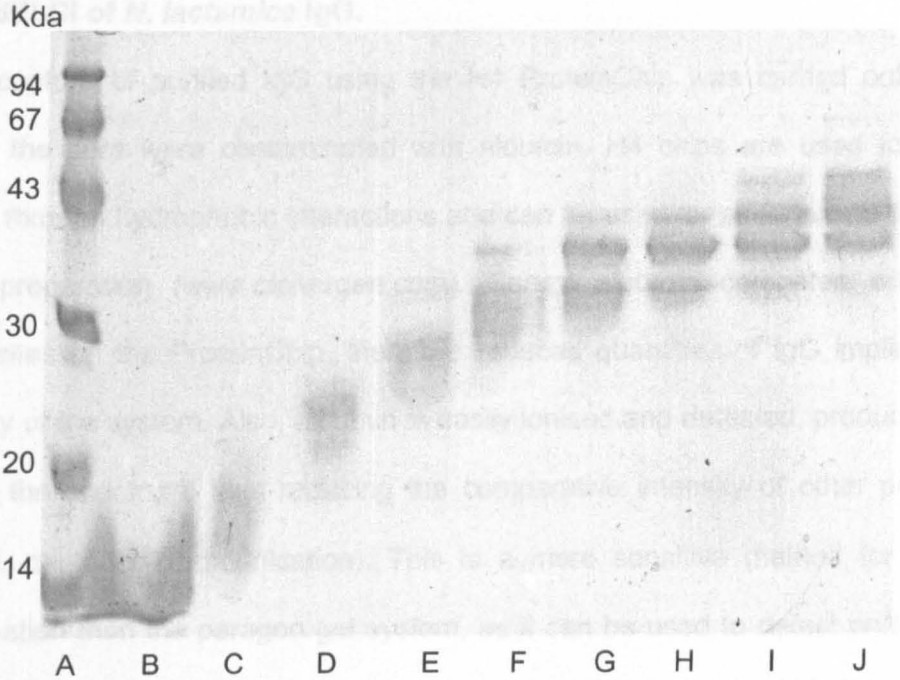

Figure 4.2 Separation of *N. meningitidis* strain MC58 cap⁻ by preparative electrophoresis. The molecular weight markers are shown in lane A. Increasing sized OMP fractions are shown in lanes B-J.

analysed on a Paragon gel (Beckman-Coulter) and no contamination of the IgG was observed (Figure 4.3). The Paragon gel consists of 1% agarose and is used for electrophoretic separation of proteins in serum. The remaining serum eluted from the column prior to IgG elution was shown to consist of albumin, alpha$_1$, haptoglobin, transferrin and complement.

4.2.3. SELDI of *N. lactamica* IgG.

Affinity capture of purified IgG using the H4 ProteinChip was carried out to assess whether the sera were contaminated with albumin. H4 chips are used for capturing proteins through hydrophobic interactions and can be used for ascertaining the purity of protein preparation (www.ciphergen.com). Serum albumin competes with IgG for binding sites on the ProteinChip, therefore reduced quantities of IgG implies reduced sensitivity of the system. Also, albumin is easily ionised and detected, producing a large peak on the spectrum, thus reducing the comparative intensity of other peaks (Paul Langford, personal communication). This is a more sensitive method for assessing contamination than the paragon gel system, as it can be used to detect proteins to the fM level. Peaks that might be expected to be seen on the mass spectra of sera would be of 22 kDa, 48 kDa, 74 kDa, 148 kDa or 66 kDa corresponding to the light chain, the heavy chain, the half antibody, intact antibody or serum albumin respectively. Half antibody (72 kDa) and intact IgG (145 kDa) could clearly be seen on the spectra of the purified IgG but a peak corresponding to serum albumin was not observed (Figure 4.4).

4.2.4. Contents of Meningococcal Protein Pools.

The molecular weights of the proteins present in each of the meningococcal OMP pools were assessed. Again H4 chips were used for affinity capture of the proteins. From the spectra, it can be seen that the pools consisted of proteins of gradually increasing mass (Figure 4.5). Pool 1 consisted of a few proteins with a maximum molecular weight of 11.2 kDa. Pool 2 consisted of proteins with masses up to 33.6 kDa. It also contained proteins over-lapping to a certain extent with those in pool 1. Pool 3 had a similar profile

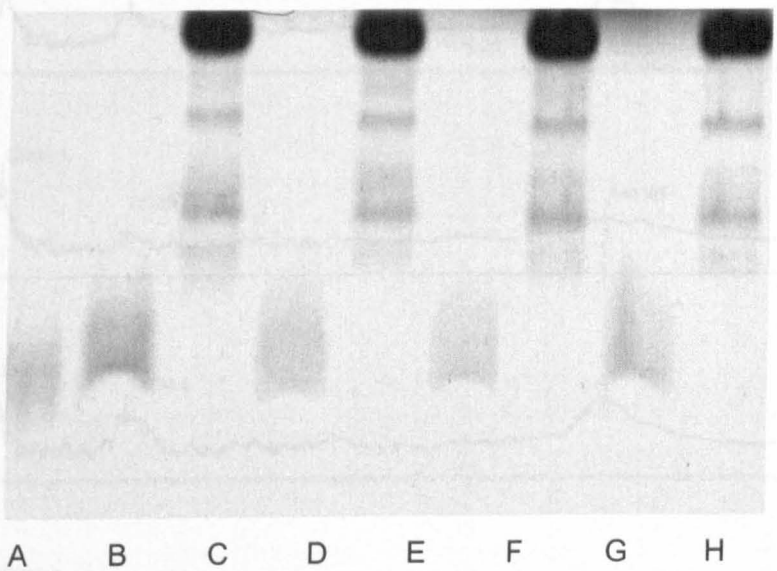

Figure 4.3 Paragon Gel showning purified IgG from *N. lactamica* antisera on lanes B, D, F and H. Lanes C, E, G and I show serum components after removal of IgG including albumin, haptoglobin, transferrin and complement. Control IgG is shown on lane A.

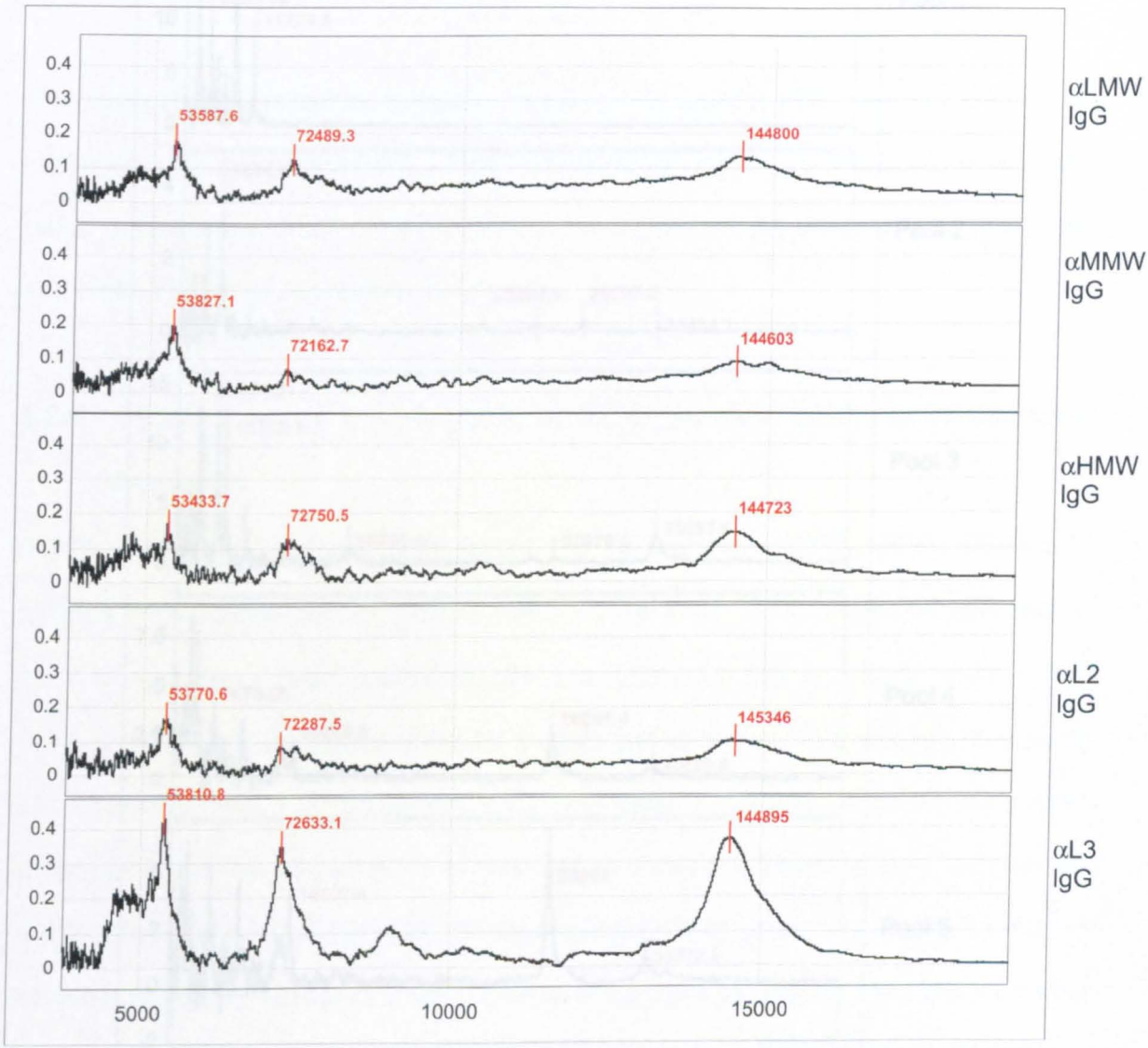

Figure 4.4 Mass spectra of rabbit sera raised against *N. lactamica* LMW, MMW, HMW, L2 and L3 outer membrane protein pools. Half antibody and intact IgG can be seen as peaks of 72 kDa and 145 kDa respectively.

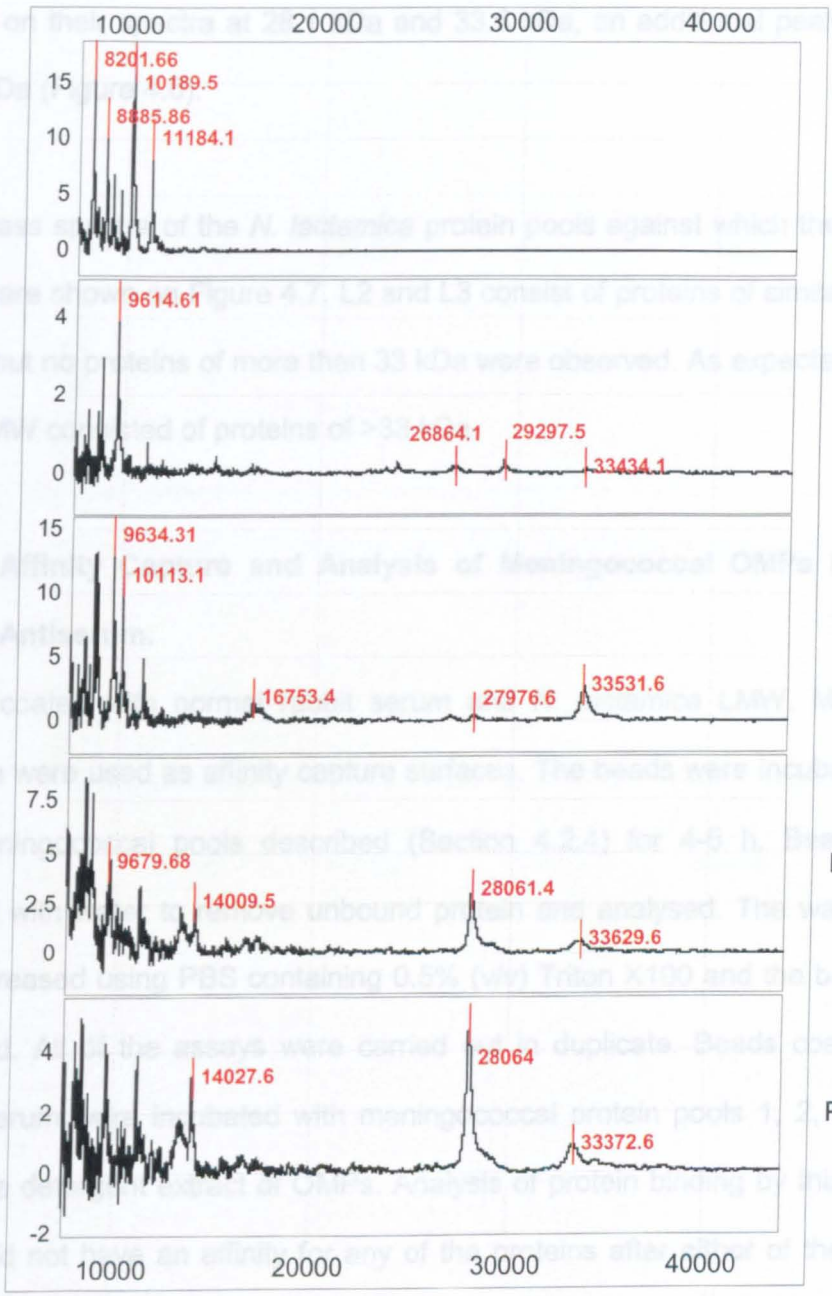

Figure 4.5 Mass spectra of meningococcal protein pools separated by preparative electrophoresis in the 5-45 kDa molecular weight range. Protein pools were bound to H4 chips and analysed.

to pool 2 but the intensities of the peaks at 26.8 kDa, 29.5 kDa and especially at 33.6 kDa were greater. Pools 4 and 5 consisted of few small proteins and as well as having peaks on their spectra at 28.1 kDa and 33.6 kDa, an additional peak was observed at 66.7 kDa (Figure 4.6).

The mass spectra of the *N. lactamica* protein pools against which the rabbit sera were raised are shown on Figure 4.7. L2 and L3 consist of proteins of similar molecular mass range but no proteins of more than 33 kDa were observed. As expected however, MMW and HMW consisted of proteins of >33 kDa.

4.2.5. Affinity Capture and Analysis of Meningococcal OMPs by *N. lactamica* Antiserum.

Beads coated with normal rabbit serum and *N. lactamica* LMW, MMW, L2 and L3 antisera were used as affinity capture surfaces. The beads were incubated with each of the meningococcal pools described (Section 4.2.4) for 4-5 h. Beads were initially washed with water to remove unbound protein and analysed. The washing stringency was increased using PBS containing 0.5% (v/v) Triton X100 and the beads were again analysed. All of the assays were carried out in duplicate. Beads coated with normal rabbit serum were incubated with meningococcal protein pools 1, 2, 3, 4, 5, and the complete detergent extract of OMPs. Analysis of protein binding by this serum showed that it did not have an affinity for any of the proteins after either of the washing steps (Figures 4.8 and 4.9).

LMW antiserum bound a number of the proteins from each of the pools (Figure 4.10). The serum bound proteins with molecular weights of approximately 11.2, 13.7, 26.9, 28.1 and 33.7 kDa from more than one protein pool when washed with water. After washing with the more stringent buffer the 11.2, 13.7 and 33.7 kDa proteins remained bound by the serum (Figure 4.11). However, the intensity of the peaks appeared to be slightly reduced. Duplicate analyses confirmed these results.

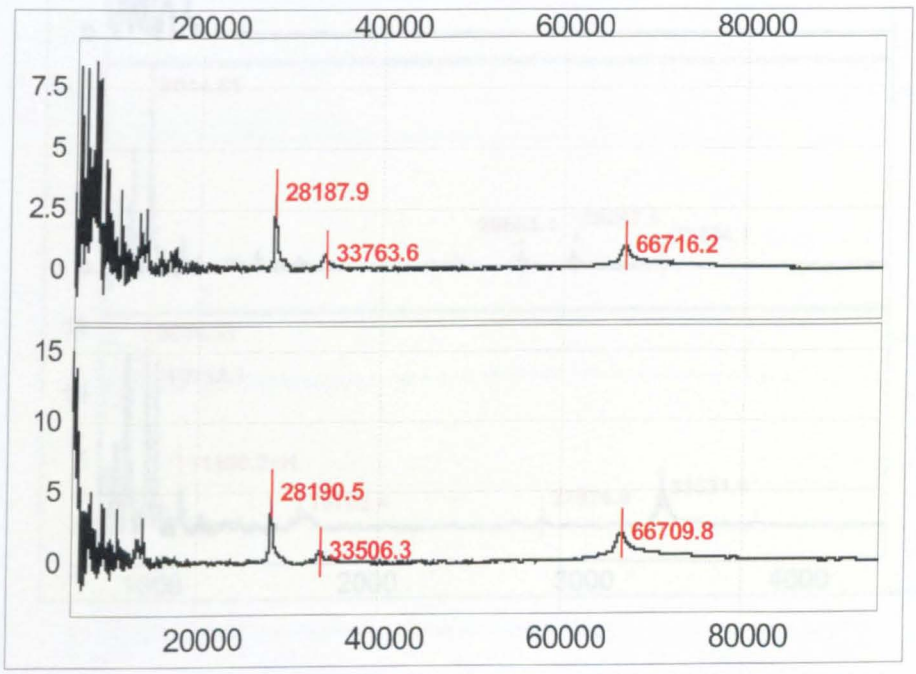

Figure 4.6 Mass spectra of *N. meningitdis* pool 4 and pool 5 in the 10-90 kDa molecular weight range.

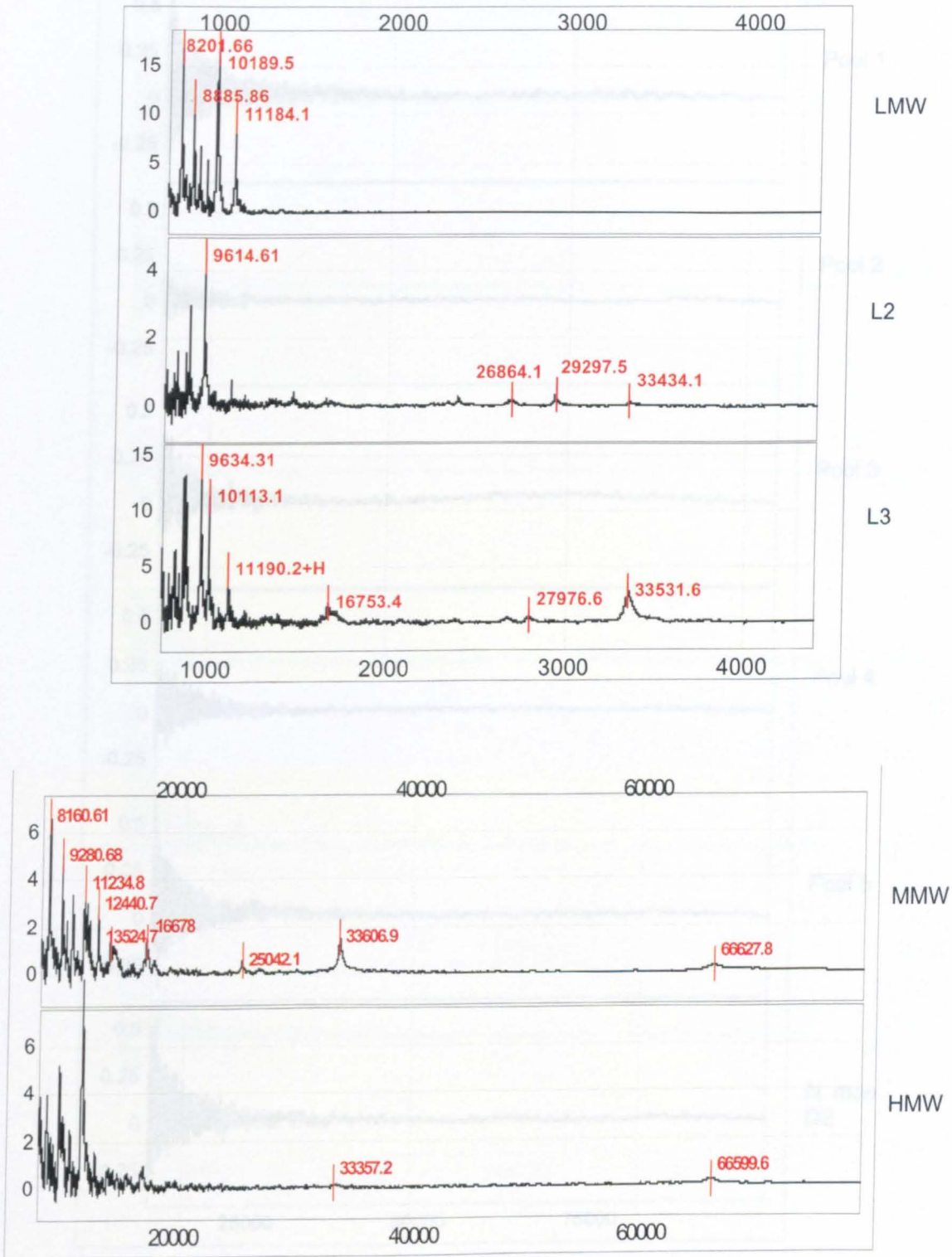

Figure 4.7 Mass spectra of *N. lactamica* protein pools against which rabbit sera were raised. LMW, L2 and L3 did not consist of proteins >33.5 kDa. MMW and HMW protein pools additionally consisted of a 66.6 kDa protein.

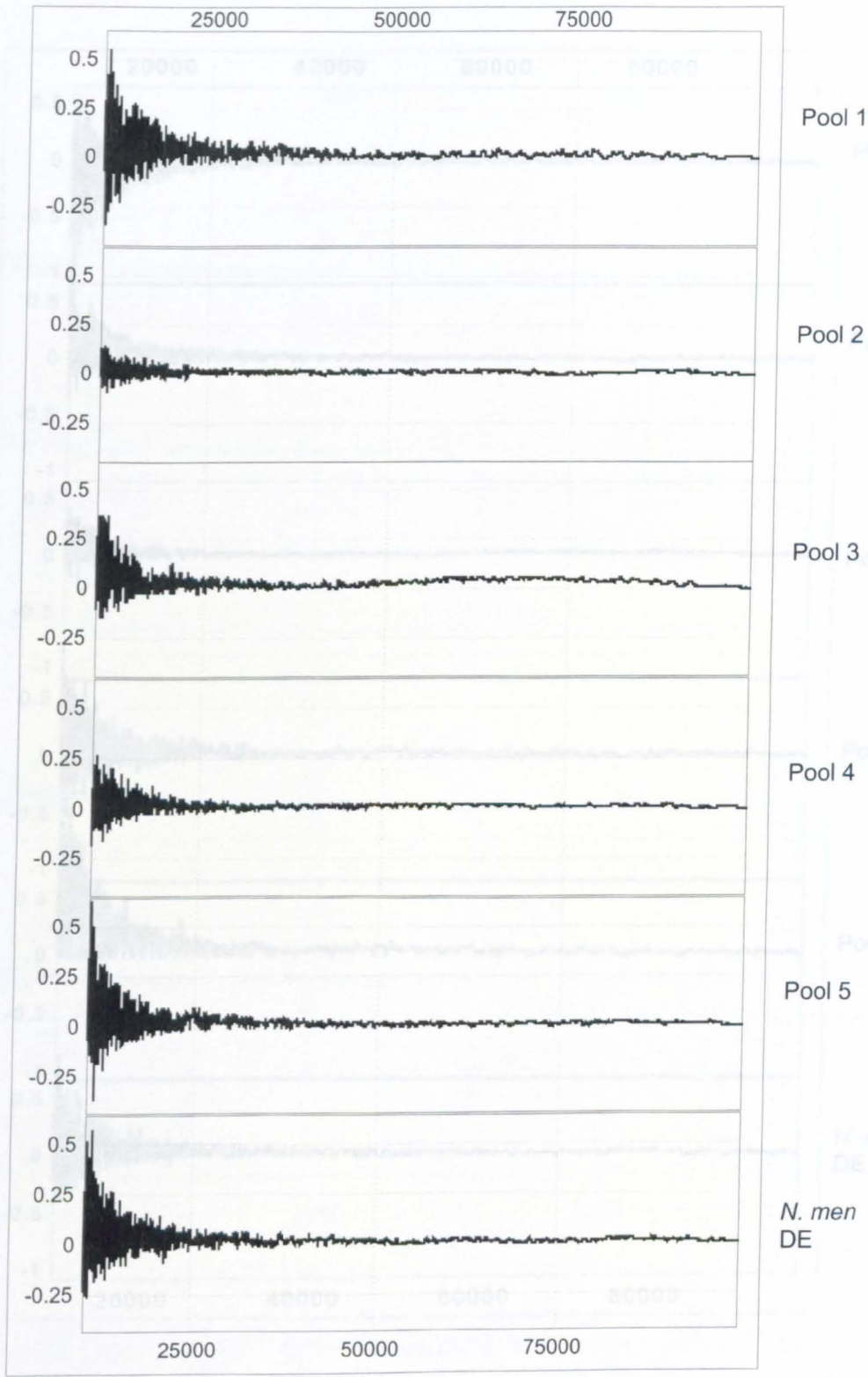

Figure 4.8 Cross-reactivity of meningococcal protein pools with normal rabbit serum after washing with water. Normal rabbit IgG was covalently bound to Dynabeads and used as control sera. Meningococcal proteins were not bound by this serum.

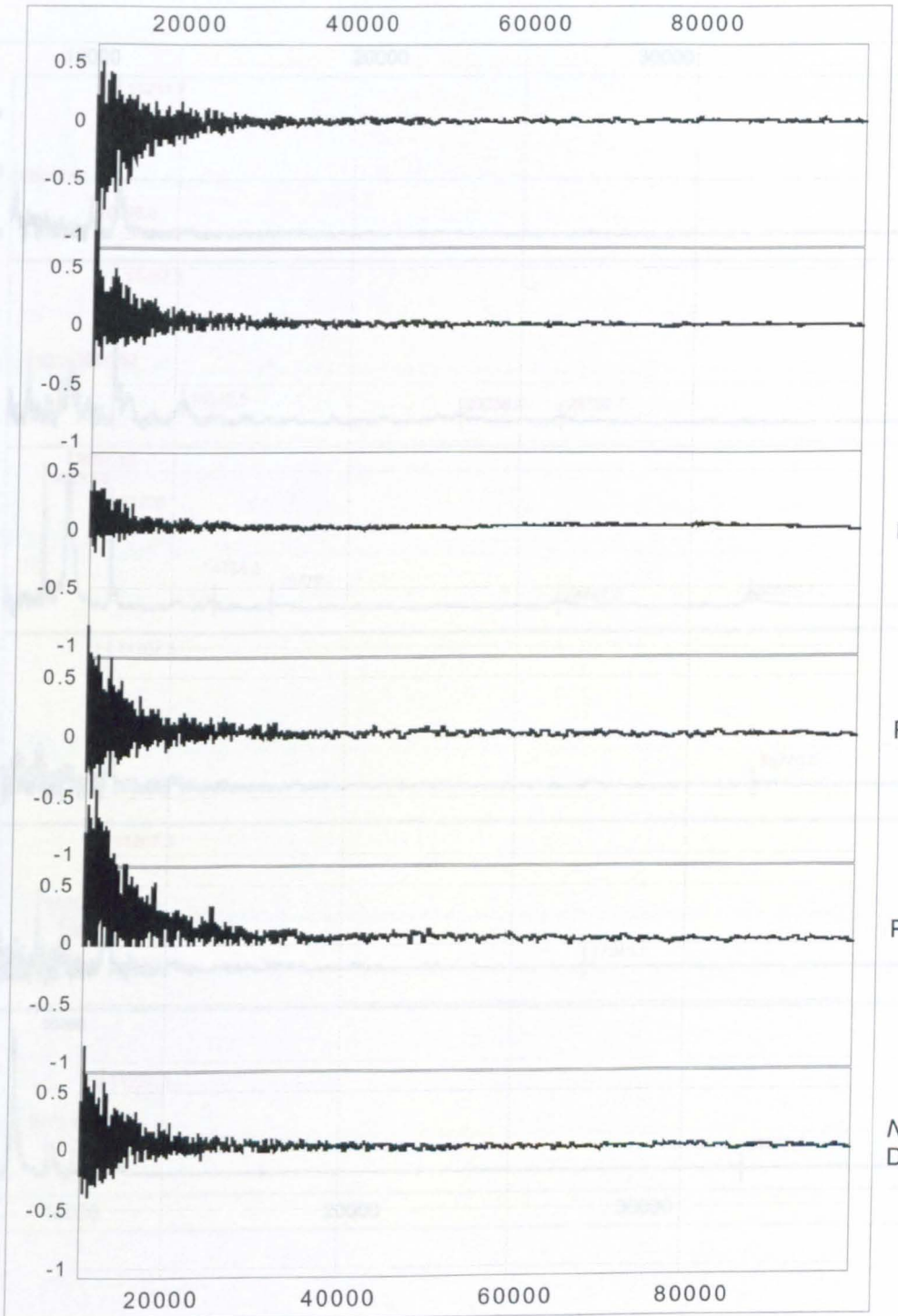

Figure 4.9 Cross-reactivity of meningococcal protein pools with normal rabbit serum after washing with PBS containing 0.5% Triton X-100.

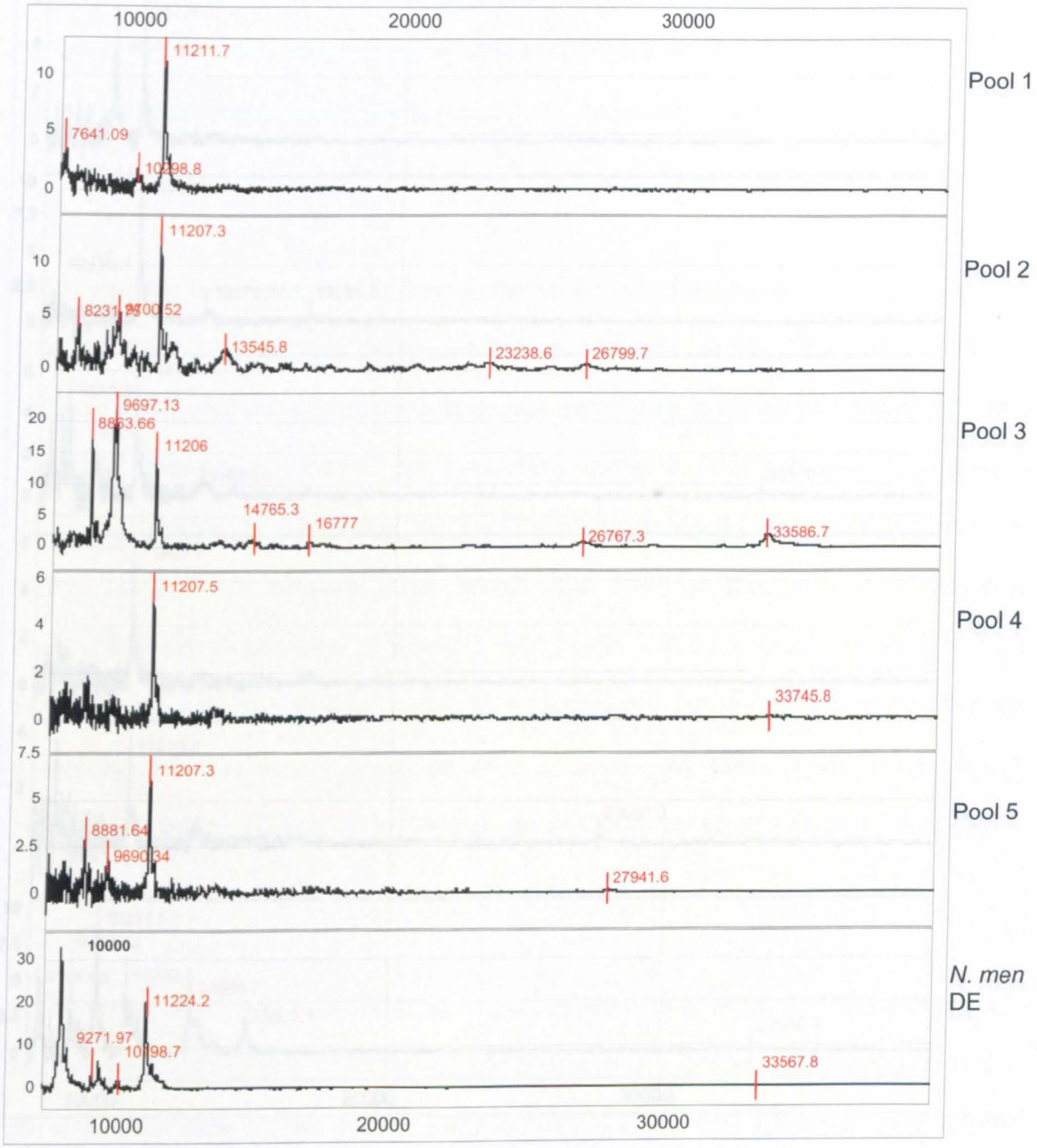

Figure 4.10 Cross-reactivity of meningococcal protein pools with LMW antiserum serum after washing with water. Anti-LMW IgG was covalently bound to Dynabeads and incubated with MC58 cap⁻ protein pools 1-5. After washing with water, a number of proteins were bound by the serum.

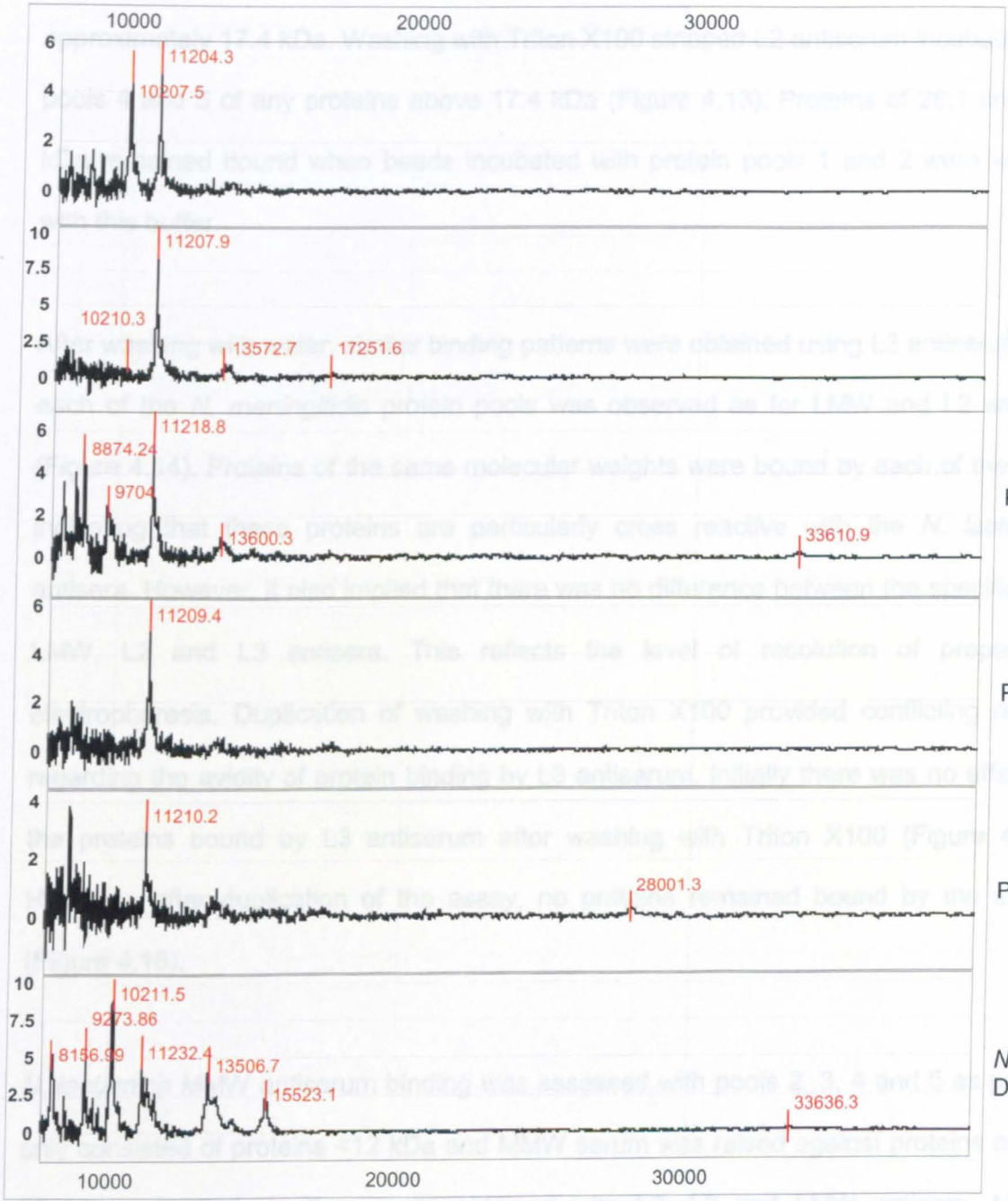

Figure 4.11 Cross-reactivity of meningococcal protein pools with LMW antiserum serum after washing with PBS containing 0.5% Triton X-100. A number of proteins remained bound by anti-LMW IgG after further washing including the 11.2, 13.7 and 33.7 kDa proteins.

L2 antiserum bound the same proteins as LMW antiserum. The protein of 28.1 kDa was only bound from protein pools 4 and 5 (Figure 4.12). Additionally, it bound a protein of approximately 17.4 kDa. Washing with Triton X100 stripped L2 antiserum incubated with pools 4 and 5 of any proteins above 17.4 kDa (Figure 4.13). Proteins of 28.1 and 33.7 kDa remained bound when beads incubated with protein pools 1 and 2 were washed with this buffer.

After washing with water, similar binding patterns were obtained using L3 antiserum with each of the *N. meningitidis* protein pools was observed as for LMW and L2 antisera (Figure 4.14). Proteins of the same molecular weights were bound by each of the sera, indicating that these proteins are particularly cross reactive with the *N. lactamica* antisera. However, it also implied that there was no difference between the specificity of LMW, L2 and L3 antisera. This reflects the level of resolution of preparative electrophoresis. Duplication of washing with Triton X100 provided conflicting results regarding the avidity of protein binding by L3 antiserum. Initially there was no effect on the proteins bound by L3 antiserum after washing with Triton X100 (Figure 4.15). However, after duplication of the assay, no proteins remained bound by the serum (Figure 4.16).

N. lactamica MMW antiserum binding was assessed with pools 2, 3, 4 and 5 as pool 1 only consisted of proteins <12 kDa and MMW serum was raised against proteins of 43-65 kDa. Similarly to the results obtained with L2, L3 and LMW antisera, MMW antiserum bound proteins of 11.2, 13.7, 17.4, 26.9, 28.1 and 33.7 kDa (Figure 4.17). Additionally, a protein of approximately 53.2 kDa from pools 2 and 4 was bound by the serum. Pools 3, 4 and 5 contained a protein of approximately 66.7 kDa which was bound by the MMW antiserum. LMW, L2 or L3 sera did not bind either the 53.2 kDa protein or the 66.7 kDa protein. Stringent washing removed any trace of the 53.2 kDa protein and often removed the 66.7 kDa protein (Figure 4.18).

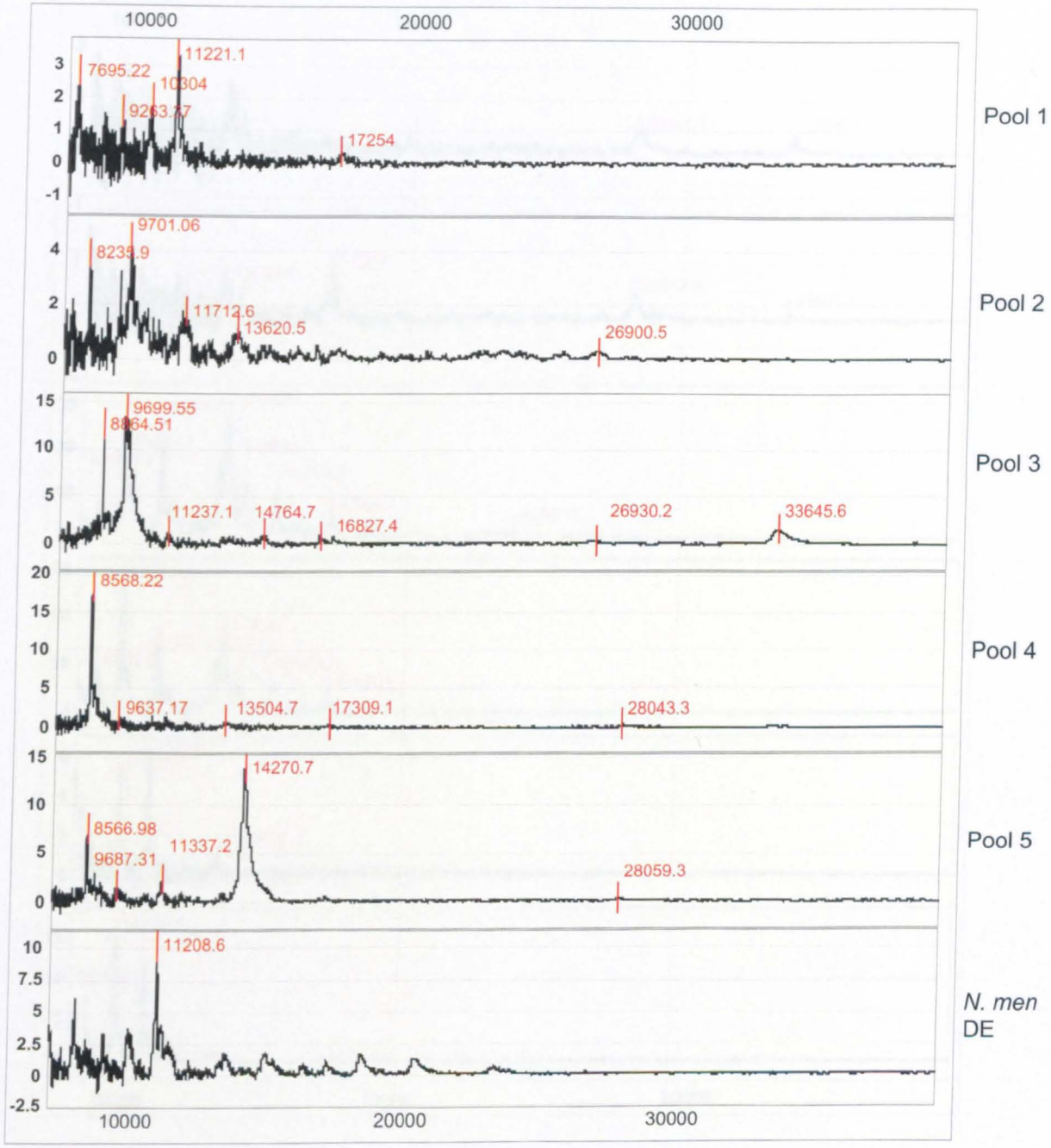

Figure 4.12 Cross-reactivity of meningococcal protein pools with L2 antiserum serum after washing with water. Anti-L2 IgG was covalently bound to Dynabeads and incubated with MC58 cap⁻ protein pools 1-5. After washing with water a similar pattern of cross-reactivity as with anti-LMW IgG was observed. A protein of 17.4 kDa was additionally bound by this serum.

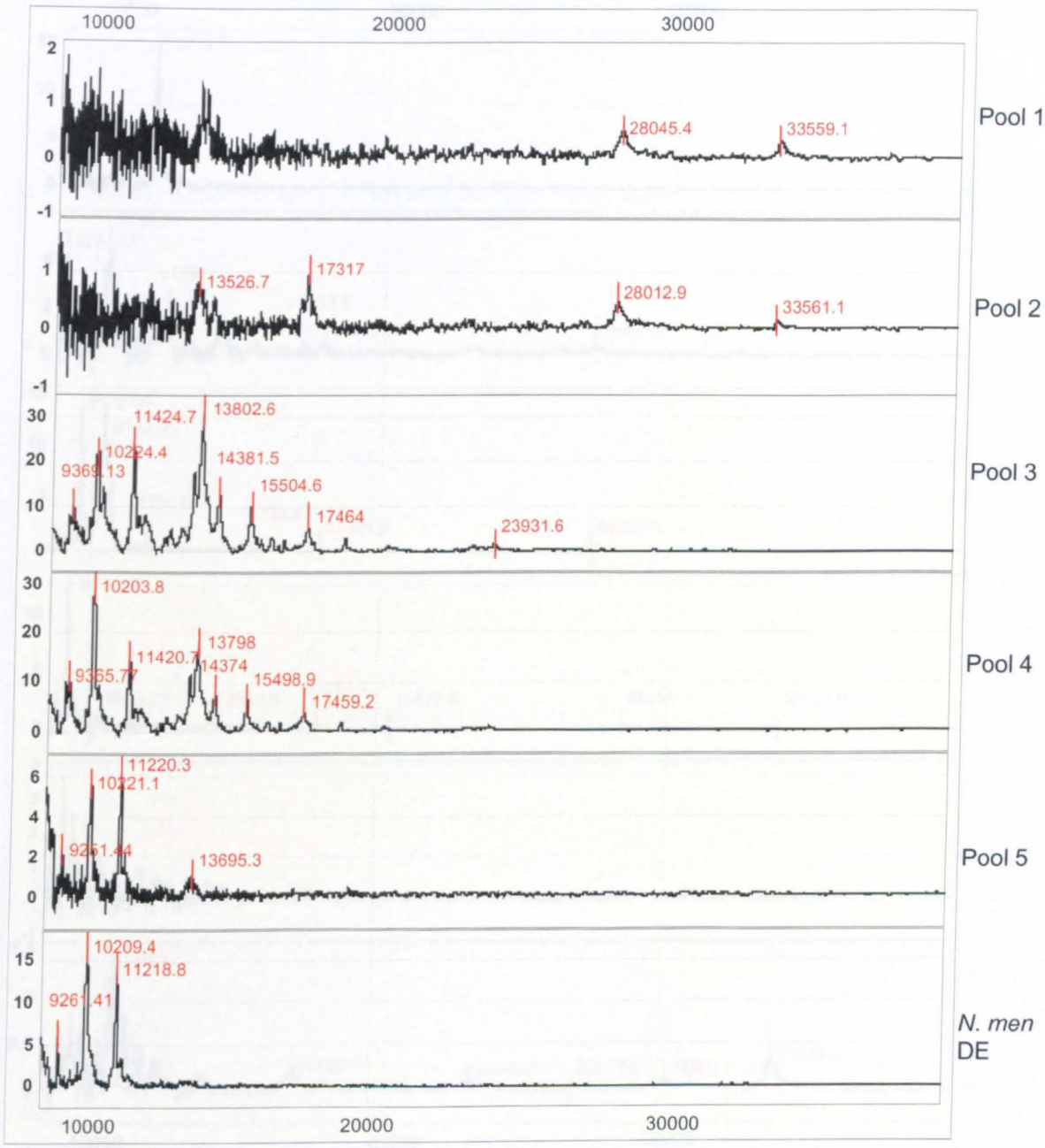

Figure 4.13 Cross-reactivity of meningococcal protein pools with L2 antiserum serum after washing with PBS containing 0.5% Triton X-100. A number of proteins remained bound by anti-LMW IgG after further washing including the 11.2, 13.7 and 33.7 kDa proteins. However, proteins of >17.4 kDa were stripped from beads incubated with MC58 cap$^-$ protein pools 4 and 5.

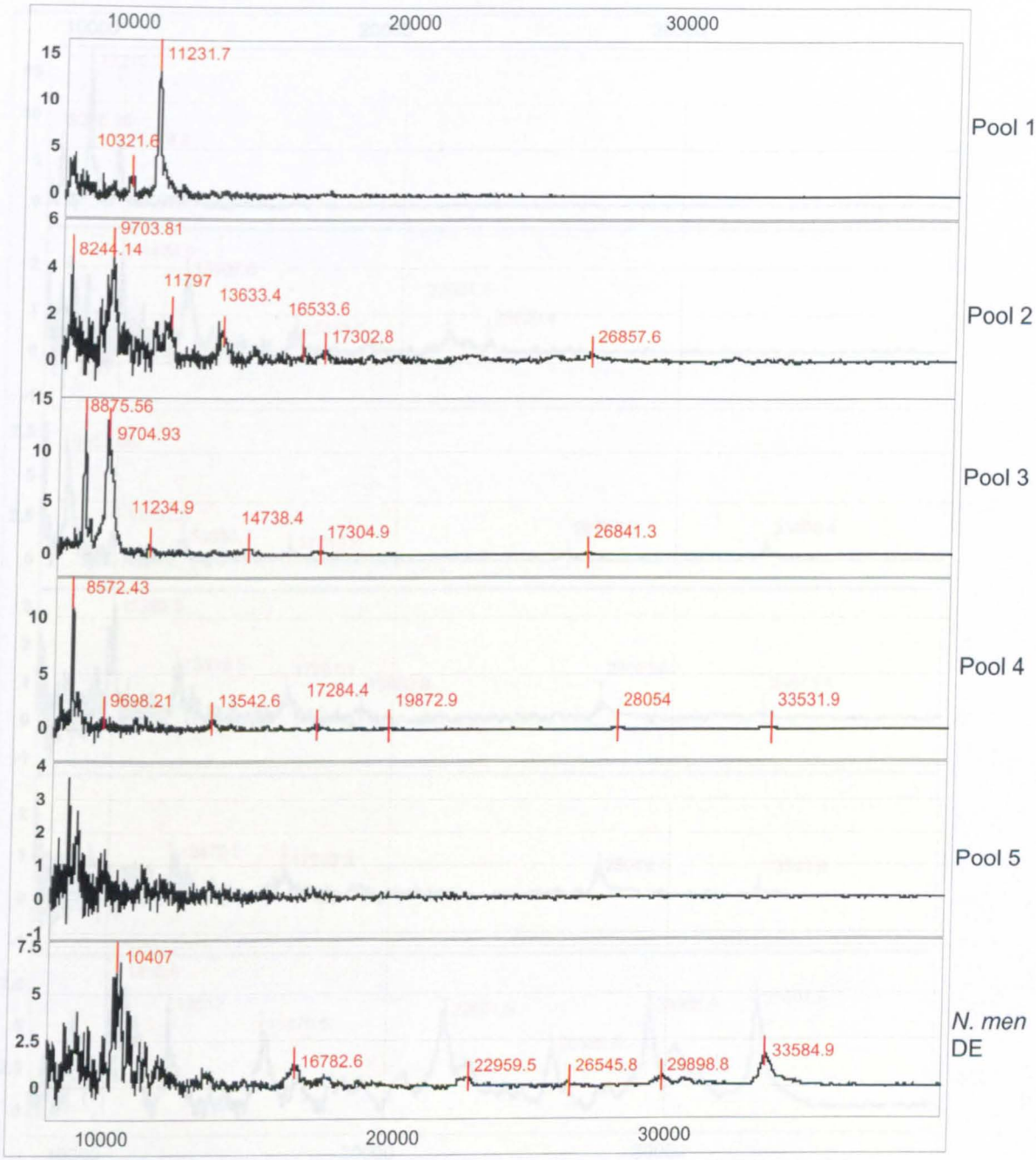

Figure 4.14 Cross-reactivity of meningococcal protein pools with L3 antiserum serum after washing with water. A similar pattern of cross-reactivity of MC58 cap⁻ protein pools was observed with Dynabeads coated with anti-L3 IgG as with beads coated with anti-LMW IgG and anti-L2 IgG.

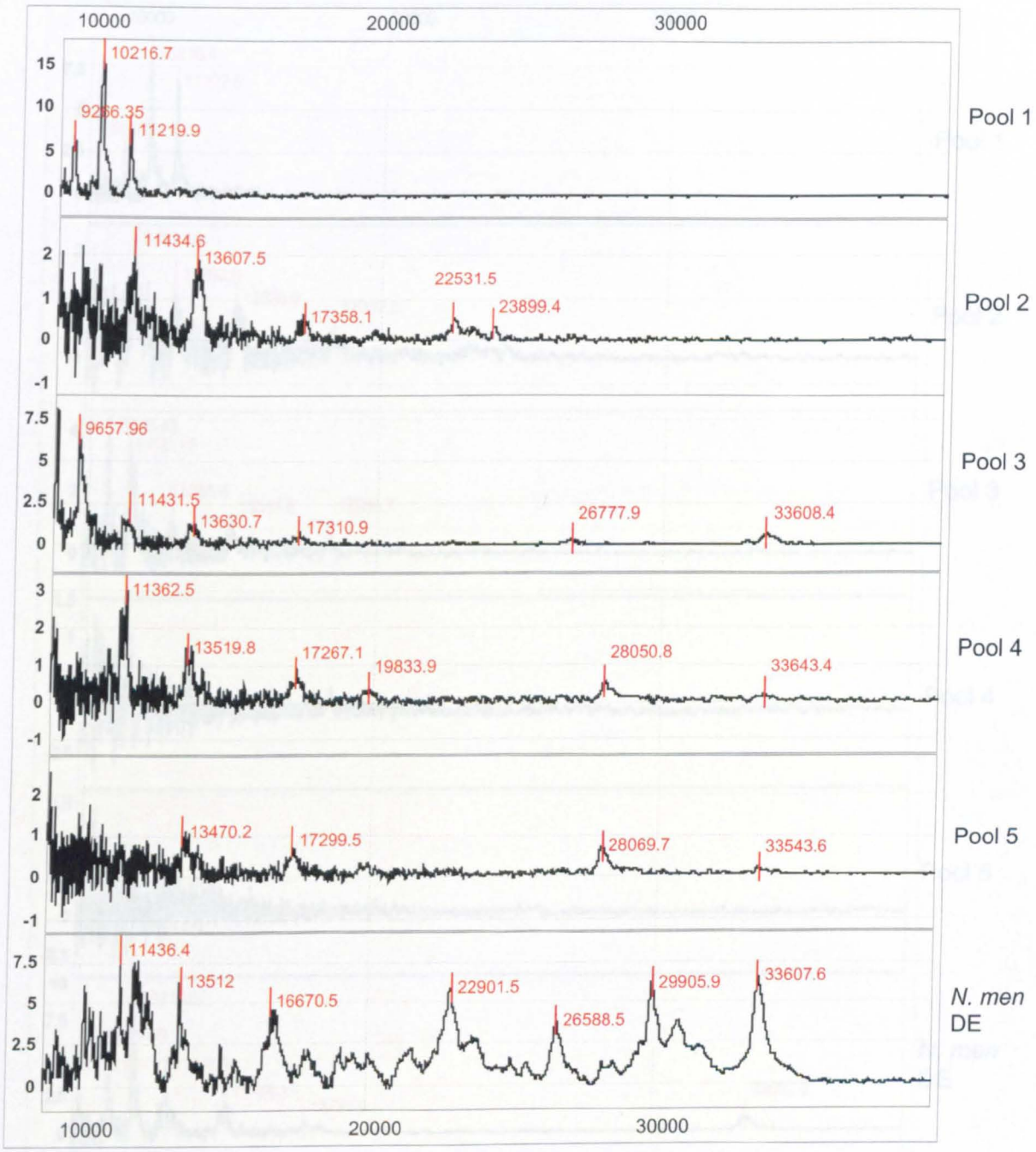

Figure 4.15 Cross-reactivity of meningococcal protein pools with L3 antiserum serum after washing with PBS containing 0.5% Triton X-100. A number of proteins remained bound by anti-LMW IgG after further washing and bound proteins were observed from MC58 cap⁻ pool 5 after this washing step.

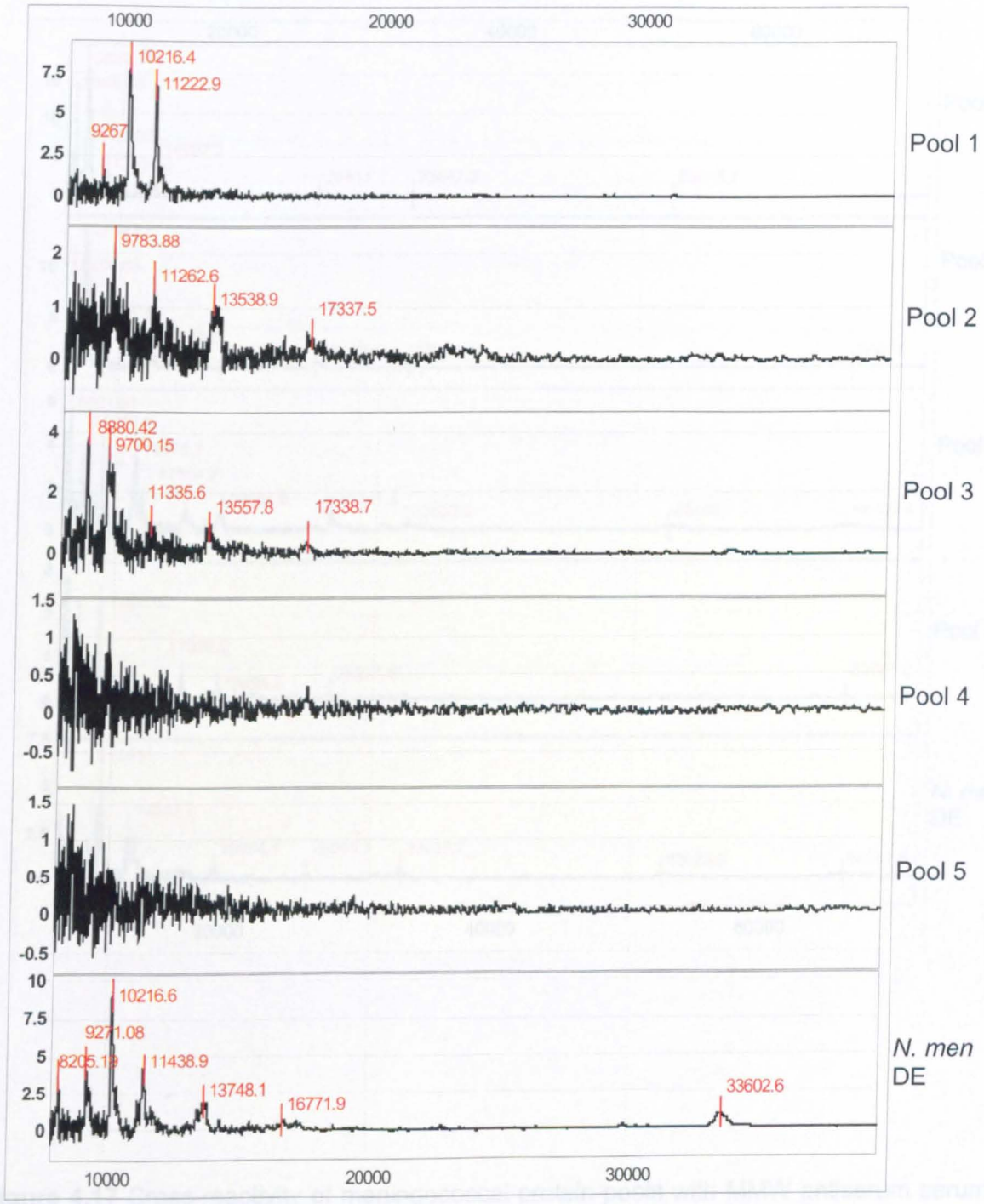

Figure 4.16 Duplicate assay showing cross-reactivity of meningococcal protein pools with L3 antiserum serum after washing with Triton X-100. No proteins from the meningococcal protein pools 4 or 5 remain bound by L3 antiserum after washing with Triton X-100. Proteins with a molecular mass greater than 17.4 kDa are also removed after this wash step from protein pools 1, 2 and 3.

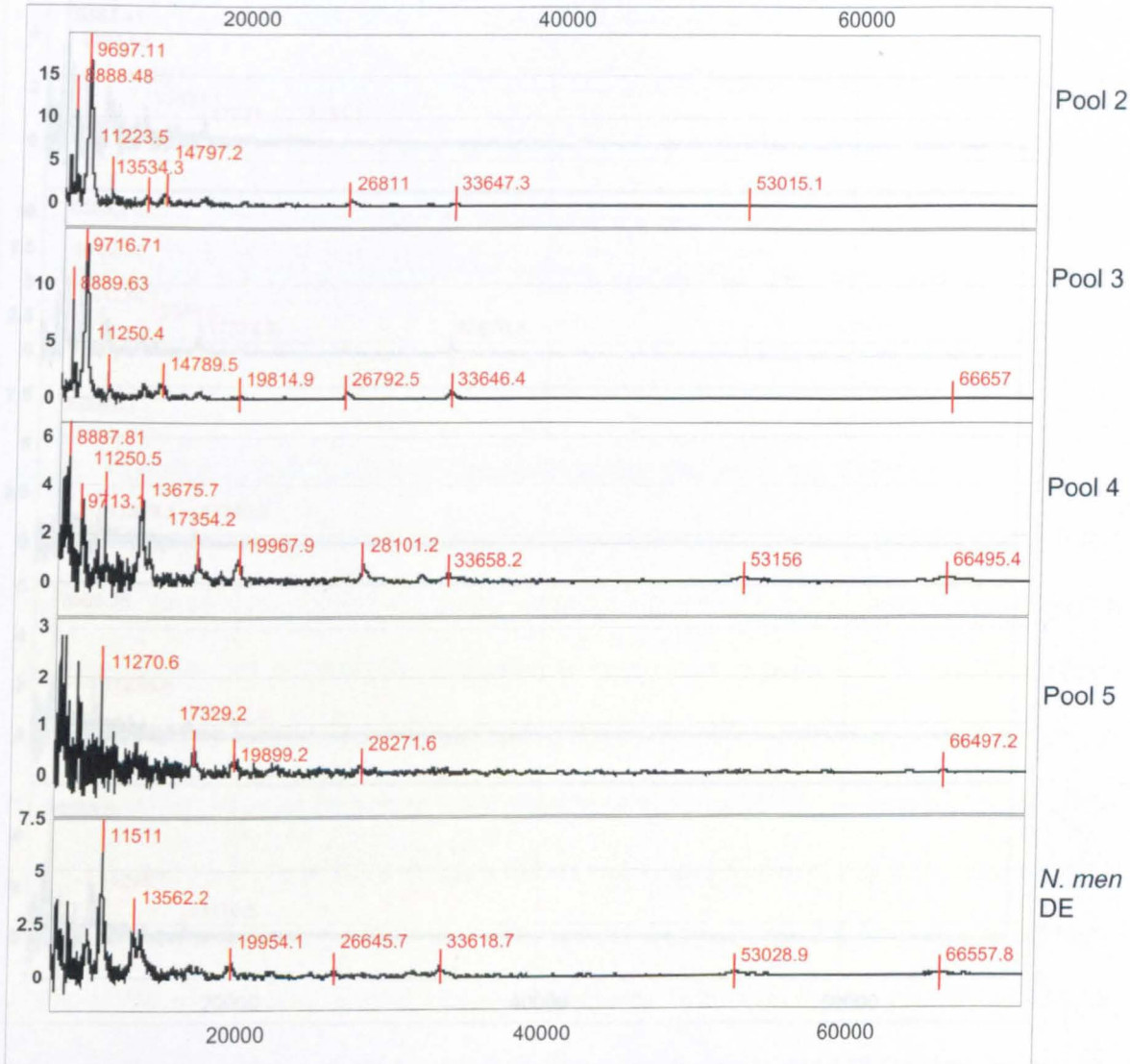

Figure 4.17 Cross-reactivity of meningococcal protein pools with MMW antiserum serum after washing with water. Anti-MMW IgG was covalently bound to Dynabeads and incubated with MC58 cap$^-$ protein pools 2-5. This serum bound similar proteins to anti-LMW IgG, anti-L2 IgG and anti-L3 IgG and additionally bound a protein of 53.2 kDa from pools 2 and 4 and a protein of 66.7 kDa fro pools 3,4 and 5 and the DE of MC58 cap$^-$.

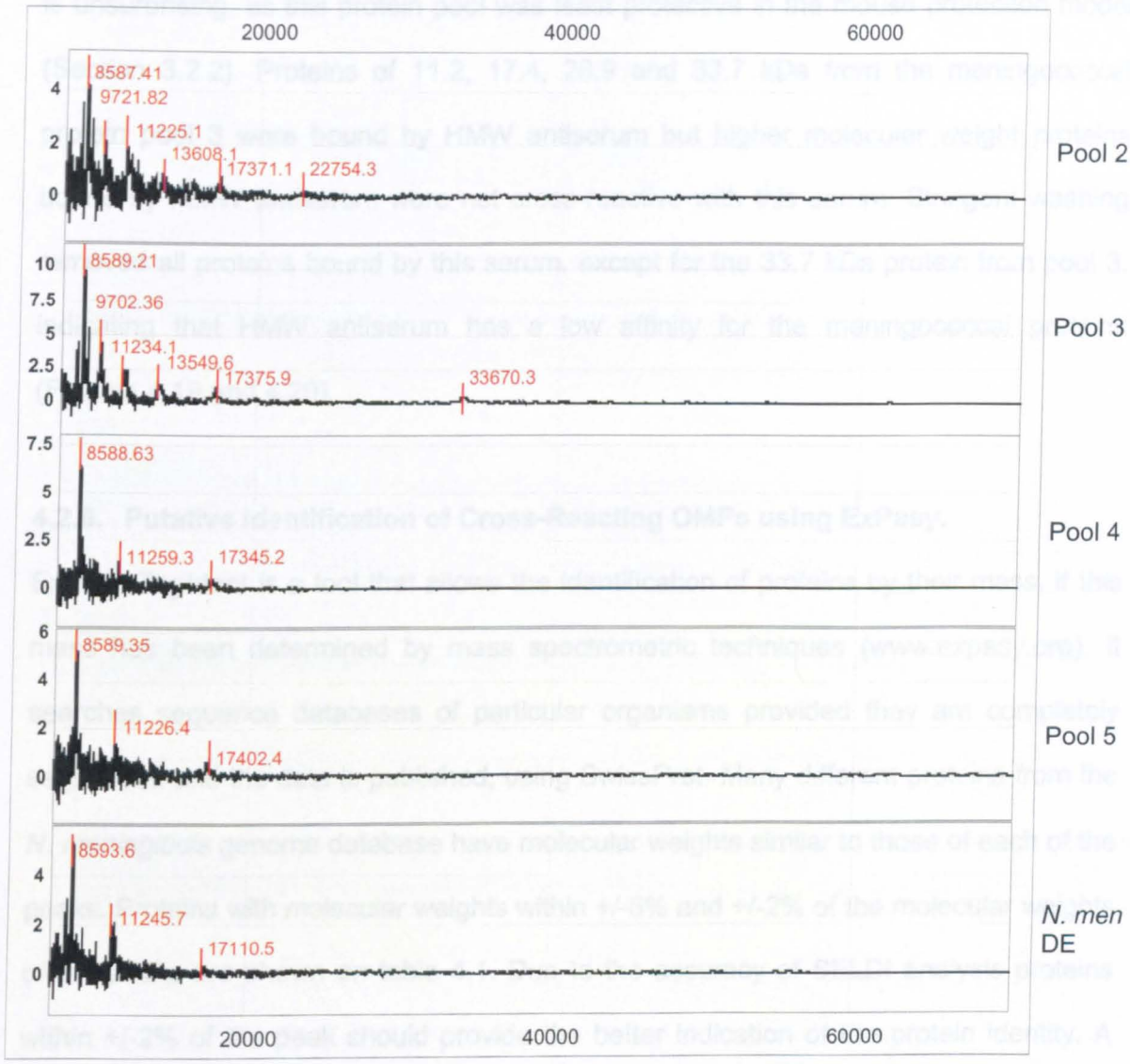

Figure 4.18 Cross-reactivity of meningococcal protein pools with MMW antiserum serum after washing with PBS containing 0.5% Triton X-100. Many proteins remained bound by anti-MMW IgG however, the 53.2 and 66.7 kDa proteins we removed from the serum after this washing step.

The proteins bound by HMW antiserum were inconsistent with the previous results. This is unsurprising, as this protein pool was least protective in the mouse protection model (Section 3.2.2). Proteins of 11.2, 17.4, 26.9 and 33.7 kDa from the meningococcal protein pool 3 were bound by HMW antiserum but higher molecular weight proteins bound by MMW antiserum were not cross-reactive with this serum. Stringent washing removed all proteins bound by this serum, except for the 33.7 kDa protein from pool 3, indicating that HMW antiserum has a low affinity for the meningococcal proteins (Figures 4.19 and 4.20).

4.2.6. Putative Identification of Cross-Reacting OMPs using ExPasy.

ExPasy TagIdent is a tool that allows the identification of proteins by their mass, if this mass has been determined by mass spectrometric techniques (www.expasy.org). It searches sequence databases of particular organisms provided they are completely sequenced and the data is published, using SwissProt. Many different proteins from the *N. meningitidis* genome database have molecular weights similar to those of each of the peaks. Proteins with molecular weights within +/-5% and +/-2% of the molecular weights of the peaks are shown on table 4.1. Due to the accuracy of SELDI analysis proteins within +/-2% of the peak should provide the better indication of the protein identity. A definitive identity of the proteins corresponding to each peak cannot be made. However, of the list of proteins within +/-2% of the peaks, the meningococcal protein with molecular weight closest to the 11.2 kDa peak was aspartate 1-decarboxylase (11.1 kDa). 50S ribosomal protein L20 (13.7 kDa) was most similar in molecular weight to the 13.7 kDa peak. Superoxide dismutase has a molecular weight of 17.36 kDa and was most similar to the molecular weight of the peak at 17.3 kDa. Hypothetical methyltransferase (27.0 kDa) was most similar in molecular weight to the 26.9 kDa peak. The molecular weight of Acyl-[acyl-carrier protein]-UDP-N-acetylglucosamine O-acetyltransferase (28.2 kDa) was most similar to the 28.2 kDa peak. The molecular weight of the meningococcal class 3 protein (33.8 kDa) was most similar to the 33.7 kDa

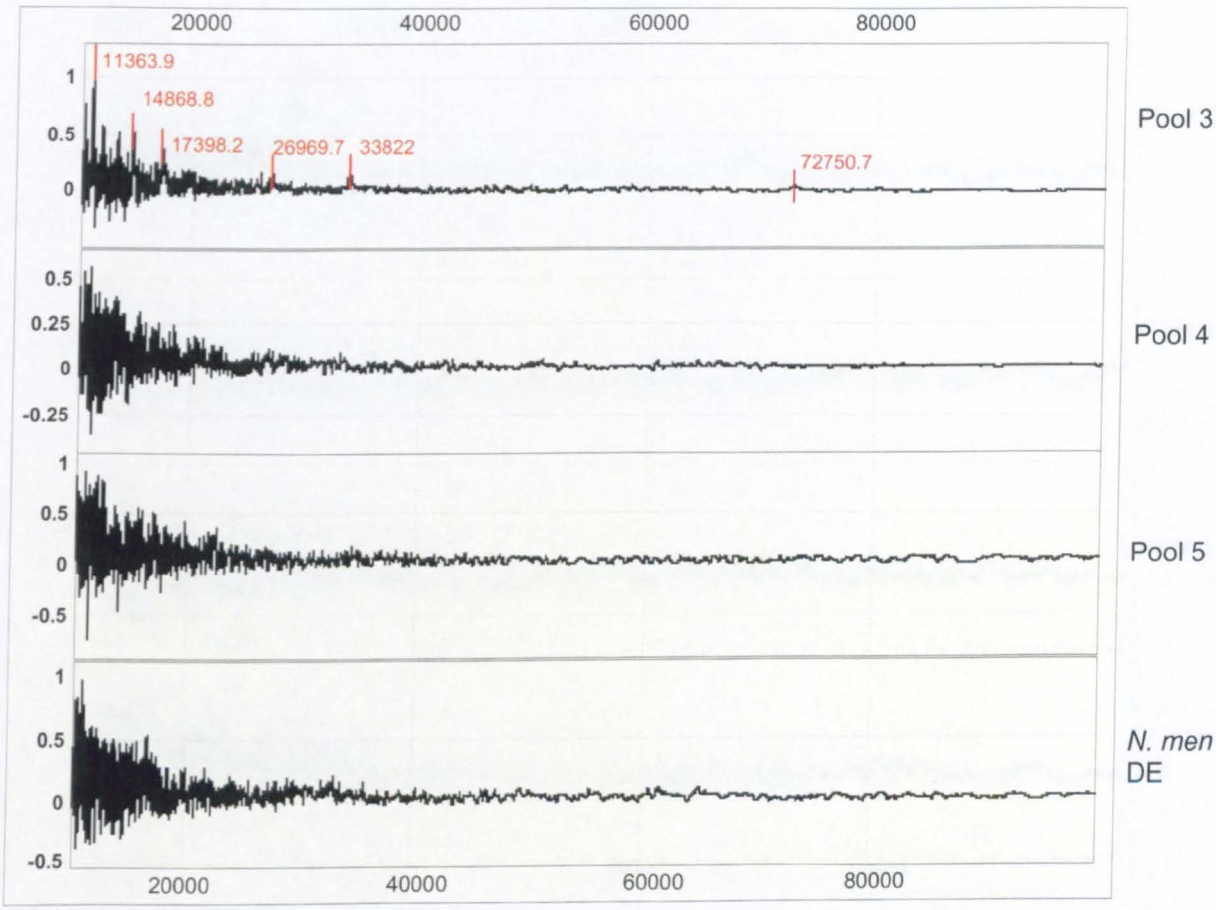

Figure 4.19 Cross-reactivity of meningococcal protein pools with HMW antiserum serum after washing with water. Anti-HMW IgG was covalently bound to Dynabeads and incubated with MC58 cap$^-$ protein pools 3-5. Only proteins from pool 3 were bound by this serum and proteins of 53.2 and 66.7 kDa were not bound from this pool.

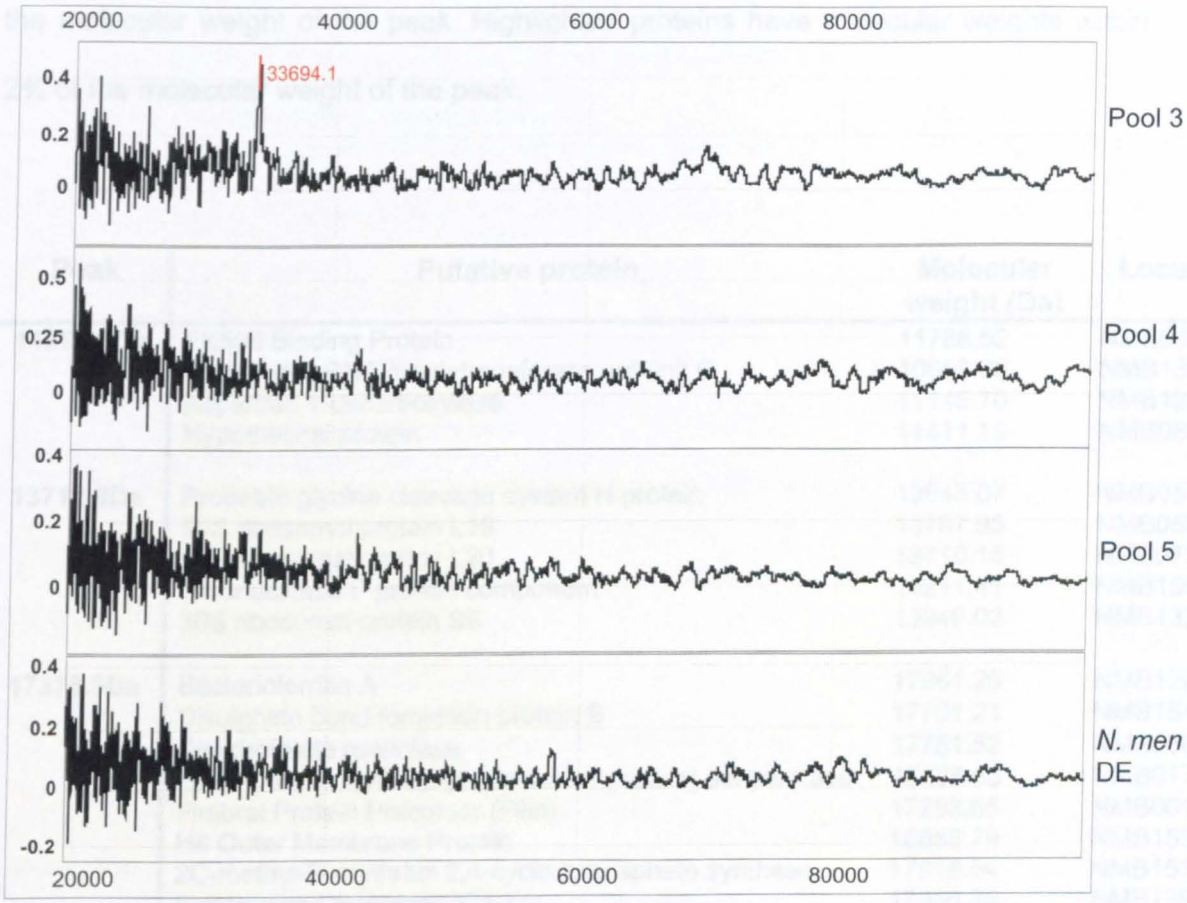

Figure 4.20 Cross-reactivity of meningococcal protein pools with HMW antiserum serum after washing with PBS containing 0.5% Triton X-100. This washing step removed all bound proteins from MC58 cap$^-$ protein pool 3 except for the 33.7 kDa protein. Anti HMW IgG had low affinity for the MC58 OMPs.

Table 4.1 Putative meningococcal proteins cross-reacting with *N. lactamica* antisera as identified using ExPasy TagIdent. All proteins listed have molecular weight within 5% of the molecular weight of the peak. Highlighted proteins have molecular weights within 2% of the molecular weight of the peak.

Peak	Putative protein	Molecular weight (Da)	Locus
11226.0Da	FK506 Binding Protein	11788.52	NMB0027
	Glutamyl T-RNA amidotransferase subunit C	10958.36	NMB1355
	Aspartate 1-decarboxylase	11145.70	NMB1282
	Hypothetical protein	11411.19	NMB0837
13712.8Da	Probable glycine cleavage system H protein	13643.07	NMB0575
	50S ribosomal protein L19	13767.95	NMB0589
	50S ribosomal protein L20	13710.15	NMB0723
	Ribonuclease P protein component	14211.41	NMB1905
	30S ribosomal protein S6	13949.02	NMB1323
17378.9Da	Bacterioferritin A	17961.25	NMB1207
	Disulphide bond formation protein B	17701.21	NMB1649
	Dihydrofolate reductase	17751.52	NMB0308
	(3R)-hydroxymyristoyl-[acyle carrier protein] dehydratase	16626.53	NMB0179
	Fimbral Protein Precursor (Pilin)	17298.65	NMB0018
	H8 Outer Membrane Protein	16885.79	NMB1533
	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	17019.54	NMB1512
	Superoxide Dismutase [Cn-Zn]	17360.36	NMB1398
	Hypothetical protein	17284.82	NMB1816
26868.0Da	3-dehydroquinate dehydratase	27186.21	NMB1446
	Acyl-[acyl-carrier protein]-UDP-N-acetylglucosamine O-acetyltransferase	28154.91	NMB0178
	Probable septum site-determining protein	26221.11	NMB0170
	Hypothetical methyltransferase	27037.62	NMB1328
	Na-translocating NADH-quinone reductase subunit C	27606.57	NMB0567
	3-methyl-2-oxobutanoate hydroxymethyltransferase	27739.25	NMB0870
	Pyridoxal phosphate biosynthetic protein	26565.58	NMB0448
	1-acyl-sn-glycerol-3-phosphate acetyltransferase	27943.25	NMB1294
	Thiazole biosynthesis protein	28067.06	NMB2071
	3-dimethylubiquinone-9 3-methyltransferase	26529.48	NMB2030
	Hypothetical protein	27417.04	NMB2054
28173.6Da	3-dehydroquinate dehydratase	27186.21	NMB1446
	Shikimate 5-dehydrogenase	28564.71	NMB0358
	Competence lipoprotein comL	29274.90	NMB0703
	Dihydrolipocolinate reductase	28328.10	NMB0203

(Table 4.1 continued)

Peak	Putative Protien	Molecular Weight (Da)	Locus
	Acyl-[acyl-carrier protein]-UDP-N-acetylglucosamine O-acetyltransferase	28154.91	NMB0178
	Hypothetical methyltransferase	27037.62	NMB1328
	Na-translocating NADH-quinone reductase subunit C	27606.57	NMB0567
	3-methyl-2-oxobutanoate hydroxymethyltransferase	27739.25	NMB0870
	1-acyl-sn-glycerol-3-phosphate acetyltransferase	27943.25	NMB1294
	Thiazole biosynthesis protein	28067.06	NMB2071
	TonB protein	29198.89	NMB1730
	Hypothetical protein	27417.04	NMB2054
33719.3Da	GTP binding protein	34617.03	NMB0678
	Glycerol-3-phosphate dehydrogenase	35337.90	NMB2060
	Porphobilinogen deaminase	33478.47	NMB0539
	33kDa chaperonin	33204.66	NMB2000
	Lacto-N-neotetraose biosynthesis glycosyl transferase	32790.00	NMB1926
	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	33986.69	NMB0017
	T-RNA delta(2)-isopentenylpyrophosphate transferase	34870.26	NMB0935
	Class 3 protein, porin	33845.23	PorB
	Proline iminopeptidase	34956.75	NMB0927
	Recombination associated protein	33263.72	NMB0851
	Glucose-1-phosphate thymidylyltransferase	32161.71	NMB0062
	Transposase for insertion sequence element IS1106	32758.54	NMB1539
	T-RNA psuedouridine synthase B	33632.50	NMB1374
	Hypothetical adenine-specific methylase	33955.36	NMB1655
66656.4Da	Chaperone protein DNA K (heat shock protein 70)	68791.66	NMB0554
	1-deoxy-D-xylulose 5-phosphate synthetase	68749.57	NMB1876
	DNA primase	65915.11	NMB1537
	Glutaminyl-T-RNA synthetase	64649.85	NMB1560
	Transferrin binding protein B	63343.98	TbpB

peak and DNA primase (65.9 kDa) was most similar in molecular weight to the 66.7 kDa peak.

4.2.7. Further Identification of the 17.3 and 66.7 kDa Proteins Peaks.

To reduce the list of putative proteins bound to the sera at the different molecular weights, mutant strains missing a protein listed or having an alteration to the protein listed rendering it unrecognisable to the serum can be used. From the list of putative proteins only superoxide dismutase C^- (SodC$^-$) and transferrin binding protein B^- (TbpB$^-$) mutant strains were available for this study. SodC, with a molecular weight of 17.36 kDa, was identified as a possible protein from the list of putative proteins as it has a molecular weight similar to the peak on the spectra at 17.3 kDa. TbpB, with a molecular weight of 63.34 kDa, is similar to the peak at 66.7 kDa, although its molecular mass is not within 2% of the peak. Comparison of binding spectra obtained with detergent extracts of wild type and mutant strains to *N. lactamica* antisera will determine whether differences can be suggested which are due to the mutation. For example, using the TbpB$^-$ mutant, the absence of a peak at 66.3 kDa, when bound by *N. lactamica* serum but the presence of the peak in the wild type strain, would indicate that this peak corresponds to TbpB. Therefore the serum may originally have been raised against *N. lactamica* TbpB, amongst other proteins, from *N. Lactamica* and it may be involved in the protection by *N. lactamica* against *N. meningitidis*.

The mutant strains used for this work were prepared by other laboratories. The TbpB$^-$ mutant of *N. meningitidis*, strain B16B6, was prepared as described by Irwin *et al.*, (1993) and the SodC$^-$ mutant of MC58 was prepared by Wilks *et al.*, (1998). The mutants were insertional, obtained by *in vitro* cassette mutagenesis.

Figure 4.21 shows the reactivity of *N. lactamica* antiserum with the strain against which the serum was raised, *N. lactamica*, strain Y92-1009, and compares the reactivity of the mutant strains with the wild type strains by western blotting. Using this method no

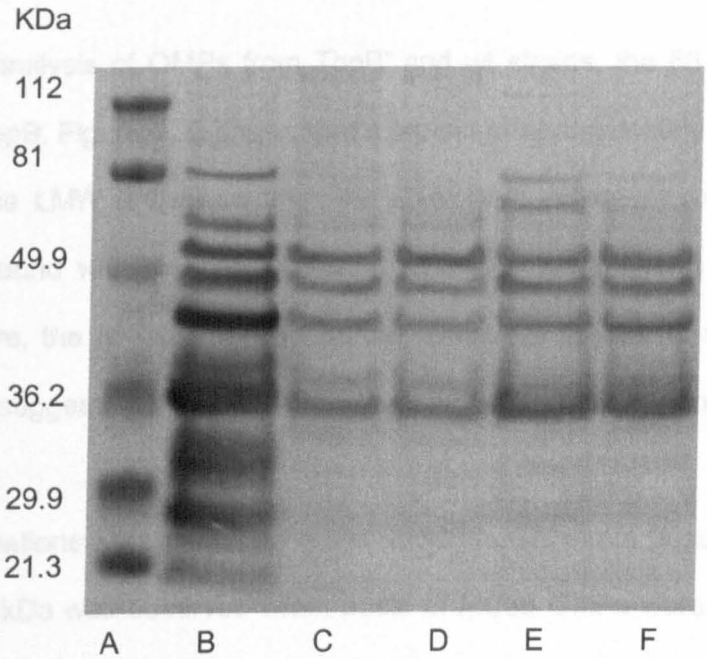

Figure 4.21 Western blot comparing cross-reactivity of *N. lactamica* LMW antiserum with TbpB- and SodC- mutant strains and their wild type parent strains. Molecular weight markers are shown on lane A. Cross-reactivity of LMW antiserum with; *N. lactamica*, strain Y92-1009, is shown on lane B; *N. meningitidis*, B16B6 wt on lane C; *N. meningitdis*, B16B6 TbpB$^-$ on lane D; *N. meningitidis*, MC58 wt on lane E; *N. meningtidis*, MC58 SodC$^-$ on lane F.

difference can be seen between the TbpB⁻ strain and the wild type strain. However, fewer high molecular weight proteins were observed in the SodC⁻ strain than the wild type strain.

Using SELDI analysis of OMPs from TbpB⁻ and wt strains, the 66.7 kDa protein was identified as TbpB. Figure 4.22 shows that a protein of approximately 66 kDa was bound by *N. lactamica* LMW antiserum from the B16B6 wt detergent extracted OMPs but nothing was bound with the corresponding molecular weight form the TbpB⁻ mutant strain. Therefore, the *N. lactamica* antiserum consisted, in part, of antibodies directed towards TbpB, suggesting that TbpB from *N. lactamica* is immunogenic.

No such observations were made using the SodC⁻ mutant strain (Figure 4.22). Although a peak of 17.3 kDa was observed when pools of MC58 OMPs were incubated with *N. lactamica* antiserum, the corresponding peak was not observed using the wild type or mutant strains to attempt to identify this peak. Therefore no conclusions can be made about the identity of the 17.3 kDa peak.

4.3. Discussion.

SELDI has been used by a number of authors as a tool for protein identification and characterisation. Hinshelwood *et al.*, (1999) used SELDI to identify the complement C3b binding site of complement factor B. Boyle *et al.*, (2001) used SELDI to analyse streptococcal virulence factor, SpeB, and its production by *S. pyogenes*. They showed that SpeB secretion was a function of cell growth and that SELDI could be used to rapidly distinguish SpeB negative and positive isogenic variants and also show the intermediates associated with SpeB production. Davies *et al.*, (1999) used SELDI to assess variants of amyloid β peptide, the component of senile plaques associated with Alzheimer's disease. A further use of MALDI which is facilitated by SELDI has been the identification of biomarkers for diagnosis of disease. For example, Johnston-Wilson *et al.*, (2001) looked for differences between samples from individuals with psychiatric

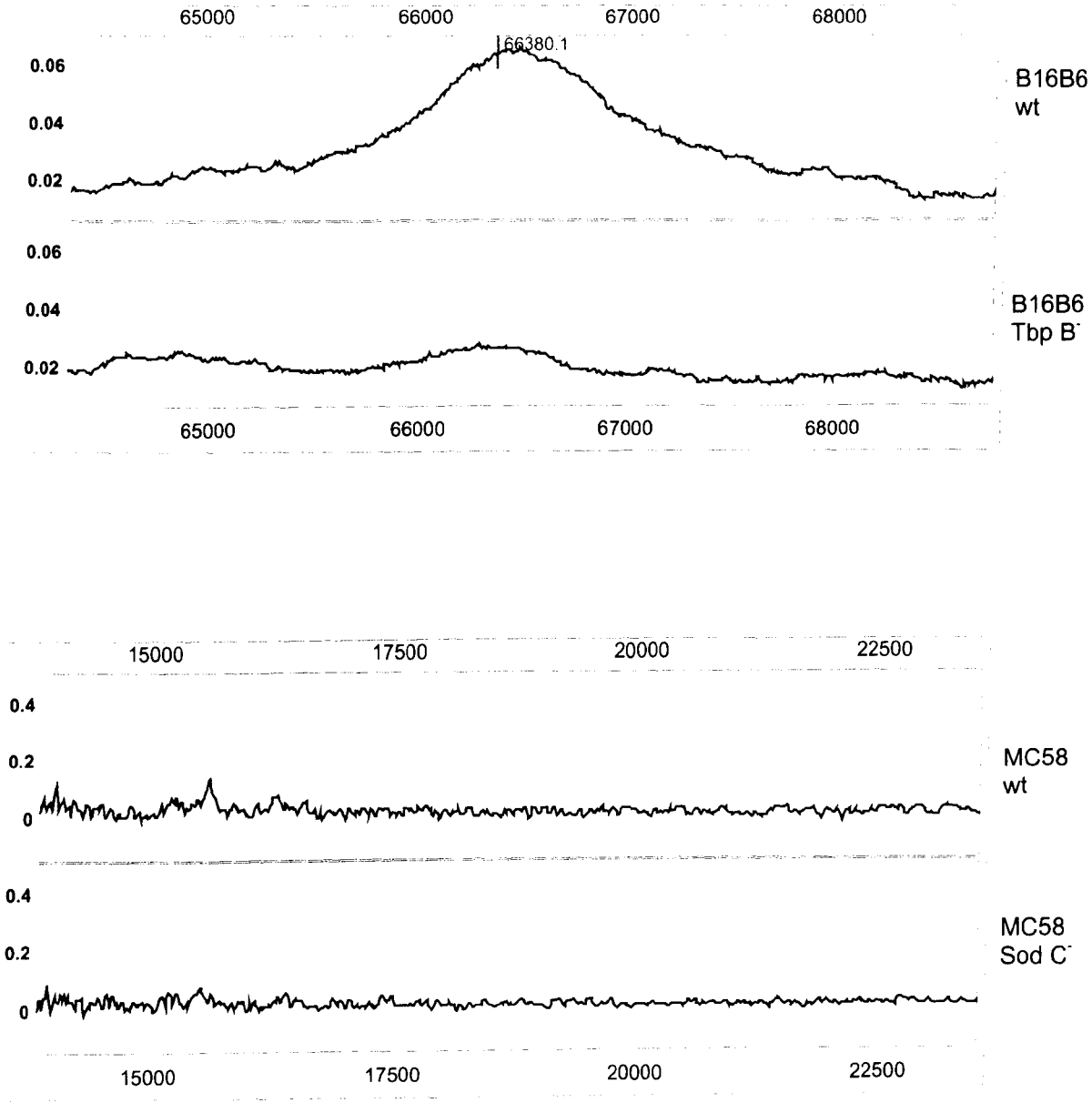

Figure 4.22 Comparison of cross-reactivity with LMW antiserum of Tbp B⁻ and Sod C⁻ meningococcal strains and their parent wild-type strains. A proteins of approximately 66 kDa from the B16B6 wt DE was bound by *N. lactamica* anti-LMW IgG but the corresponding protein in the mutant strain was not observed implying that this protein peak corresponds to TbpB. Results using MC58 wt and SodC⁻ strains were inconclusive.

disease and controls. SELDI has also been used by Kuwata *et al.*, (2001) to observe the products of digestion of lactoferrin. However, no work prior to this study has used SELDI as a tool for observing the cross reactivity of complex mixtures of proteins with serum raised against complex mixtures of proteins from another species and to identify these proteins.

In this study, sera were raised in rabbits against OMPs from *N. lactamica* that were previously shown to be protective in mice (chapter 3, section 3.2.2). The cross-reactivity of these sera with OMPs from *N. meningitidis* was assessed using SELDI. Proteins with molecular weights of approximately 11.2, 13.7, 17.4, 26.9, 28.1, 33.7, 53.2 and 66.7kDa were most cross-reactive with the range of antisera tested, suggesting that proteins homologous to these from *N. lactamica* are important in mounting a cross-reactive immune response against *N. meningitidis*. Putative identifications of these proteins were made by comparing the molecular masses of the proteins with the published proteome of *N. meningtidis*, strain MC58.

4.3.1. SELDI as a Tool for Protein Identification.

The molecular masses of the proteins bound by *N. lactamica* sera were compared to the masses of proteins from the proteome of *N. meningitidis* using ExPasy TagIdent and searching the SwissProt database. A number of putative proteins were identified, corresponding to each of the bound proteins. However, the data was ambiguous; there were no putative proteins with molecular weights exactly matching the mass of the bound protein. As a best guess of identity, the putative protein closest in molecular weight to the bound protein could provide the best identity. Alternatively, as sera were raised against *N. lactamica* OMPs and the meningococcal protein pools consisted of OMPs, some proteins may have a slightly different mass between *N. meningtidis* and *N. lactamica* homologues. We could also assume that the putative proteins should be transmembrane. Transmembrane prediction packages, such as TMpred, proteins can predict that proteins have a transmembrane location. However, it uses a set of rules

based on naturally occurring transmembrane proteins to predict this, which may not necessarily apply to the protein of interest. For this study, transmembrane proteins among the putative proteins identified were predicted (Table 4.2). This still left a long list of putative proteins for each of the bound proteins except for the proteins of 11.2 and 13.7 kDa, where none of the putative proteins were predicted to be transmembrane. Predicting transmembrane proteins among the putative proteins did not provide enough information to aid identification of the cross-reactive proteins. Also transmembrane prediction does not automatically imply the protein is an OMP as the proteins may be bound to an intracellular membrane. Finally, to identify the cross-reactive proteins, mutant strains missing the protein of interest were used. Serum binding of OMPs from the mutant strain and the wt strain were compared using SELDI. Using this method we were able to show that the 66.7 kDa protein was probably TbpB. However, to predict with certainty the identity of the 66.7 kDa protein, other strains with mutations in the remaining proteins from the list corresponding to the protein of 66.7 are required. Using the SodC mutant strain to identify the 17.4 kDa protein produced ambiguous results. The use of mutant strains is limited unless the strains used to prepare the mutant are the same as the wt strain being tested for binding. In this case the TbpB$^-$ strain was B16B6, but initial assessment of binding to *N. lactamica* antisera was carried out with pools of *N. menigntidis*, strain MC58, OMPs. Better identification of meningococcal proteins bound by *N. lactamica* antisera would have been made if the meningococcal proteins could have been purified and binding of individual proteins assessed. Individual cross-reactive proteins could have been cleaved into peptide fragments and the true identity of the protein found.

MS facilitates the identification of proteins and a number of authors discuss the use of 2-dimensional (2D) electrophoresis and cleavage of individual proteins of interest to obtain a set of peptides corresponding to the protein as a preliminary step to protein identification prior to MS. Peptide mass fingerprinting is the most commonly used method for identification of proteins using MS. The protein of interest is enzymically or

Table 4.2 TMpred Transmembrane Prediction of Putative Proteins Identified using ExPasy. Transmembrane prediction does not automatically imply that the protein is an outer membrane protein. **X** means that the protein is not predicted to be transmembrane. ✓ means that the protein is predicted to be transmembrane.

Peak	Putative protein	T/M (X/✓)
11226.0Da	FK506 Binding Protein	X
	Glutamyl T-RNA amidotransferase subunit C	X
	Aspartate 1-decarboxylase	X
	Hypothatical protein	X
13712.8Da	Probable glycine cleavage system H protein	X
	50S ribosomal protein L19	X
	50S ribosomal protein L20	X
	Ribonuclease P protein component	X
	30S ribosomal protein S6	X
17378.9Da	Bacterioferritin A	X
	Disulphide bond formation protein B	✓
	Dihydrofolate reductase	✓
	(3R)-hydroxymyristoyl-[acyle carrier protein] dehydratase	✓
	Fimbral Protein Precursor (Pilin)	✓
	H8 Outer Membrane Protein	✓
	2C-methyl-D-erythritol 2,4-cyclodiphosohate synthase	✓
	Superoxide Dismutase [Cn-Zn]	✓
	Hypothetical protein	X
26868.0Da	3-dehydroquinate dehydratase	✓
	Acyl-[acyl-carrier protein]-UDP-N-acetylglucosamine O-acetyltransferase	✓
	Probable septum site-determining protein	X
	Hypothetical methyltransferase	X
	Na-translocating NADH-quinone reductase subunit C	✓
	3-methyl-2-oxobutanoate hydroxymethyletransferase	X
	Pyridoxal phosphate biosynthetic protein	X
	1-acyl-sn-glycerol-3-phosphate acetyltransferase	✓
	Thiazole biosynthesis protein	✓
	3-demethylubiquinone-9 3-metyltransferase	X
	Hypothetical protein	X
28173.6Da	3-dehydroquinate dehydratase	✓
	Shikimate 5-Dehydrogenase	✓
	Competence lipoprotein comL	✓
	Dihydrolipocolinate reductase	✓

(Table 4.2 continued)

Peak	Putative Protien	T/M (X/✓)
	Acyl-[acyl-carrier protein]-UDP-N-acetylglucosamine O-acetyltransferase	✓
	Hypothetical methyltransferase	X
	Na-translocating NADH-quinone reductase subunit C	✓
	3-methyl-2-oxobutanoate hydroxymethyletransferase	✓
	1-acyl-sn-glycerol-3-phosphate acetyltransferase	✓
	Thiazole biosynthesis protein	✓
	TonB protein	✓
	Hypothetical protein	X
33719.3Da	GTP binding protein	X
	Glycerol-3-phosphate dehydrogenase	✓
	Porphobilinogen deaminase	X
	33kDa chaperonin	X
	Lacto-N-neotetraose biosynthesis glycosyl transferase	X
	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	X
	T-RNA delta(2)-isopentenylpyrophosphate transferase	✓
	Class 3 protein, porin	✓
	Proline iminopeptidase	✓
	Recombination associated protein	X
	Glucose-1-phosphate thymidylyltransferase	✓
	Transposase for insertion sequence element IS1106	X
	T-RNA psuedouridine synthase B	X
	Hypothetical adenine-specific methylase	✓
66656.4Da	Chaperone protein DNA K (heat shock protein 70)	X
	1-deoxy-D-xylulose 5-phosphate synthetase	✓
	DNA primase	✓
	Glutaminyl-T-RNA synthetase	X
	Transferrin binding protein B	✓

chemically cleaved and the peptide mixture produced by cleavage is analysed by MS. The peptide mass spectrum is compared to spectra obtained by theoretical cleavage of proteins sequences stored in databases. Only a small number of accurately measured peptide masses are required for protein identification and proteins in fM concentrations can be identified from 2D gels (Gevaert *et al.*, 2000). Peptide analysis was carried out after 2D electrophoresis of *Escherichia coli* OMPs (Molloy *et al.*, 2000). 2D electrophoresis gave information regarding the pI of the proteins prior to cleavage, which was used reduce the number of outcomes when searching for the peptide identity.

However, 2D-electrophoresis is a labour-intensive and time-consuming process. Many large or hydrophobic proteins do not enter the gel so a complete picture of the proteins that constitute the entire proteome of an organism is not obtained (Graves and Haystead, 2002). Low copy proteins are also problematic to detect and the gel cannot be overloaded because the resolution of the gel will decrease and co-migration of proteins will increase. Therefore, less complex mixtures of proteins are best for analysis by 2D-electrophoresis. However a protein mixture of reduced complexity was used in this chapter therefore 2D separation of these proteins may have proved useful. A further problem with the use of 2D-electrophoresis prior to MS is that proteins cannot be extracted efficiently from gels. However, the protein of interest can be digested whilst in the gel and peptides extracted more efficiently. Analysis of the peptide profile of a protein by MS provides more information about the protein than can be obtained from the mass of the whole protein. Using MALDI or SELDI the peptide mixture can be analysed directly, without purification. The main problem with peptide analysis is that although the mass measured by MS is accurate there is ambiguity in peptide identification as a peptide can be of the same mass, independent of the order of its constituent amino acids. The masses of a large number of the peptides of a cleaved protein must be obtained to achieve the correct identity of the protein. Also, this technique is not suitable for mixtures of even 2 proteins, as the peptide mixture of the

proteins would be very complex (Graves and Haystead, 2002), therefore the constituent proteins of the protein pools used in this study would have to be purified, probably by 2D electrophoresis. However, Park and Russell (2001) have recently used MS to identify protein constituents from complex mixtures. Synthetic protein mixtures were prepared and the mixture was subjected to thermal denaturation and then tryptic digestion. The peptides were analysed by MALDI and the digest fragments identified.

4.3.2. Benefits of SELDI Compared with Western Blotting.

Western blotting provided little information about the identity of the meningococcal proteins from the wt and mutant strains cross-reactive with *N. lactamica* antiserum. The molecular weights of the cross-reactive proteins were approximate, unlike SELDI where the accuracy is 0.1%. Molecular weights are determined after separation by comparison of the migration of the proteins to a set of protein standards. The accuracy of protein molecular weight determination by SDS-PAGE, and therefore western blotting, ranges from between a few percent up to 30% (Jardine, 1990). Burnette (1981) first described Western blotting as a technique for immunological detection. The molecular weight of the protein affects the rate of transfer of the protein. Higher molecular weight proteins are transferred less quickly than low molecular weight proteins. Therefore, the accuracy of western blotting is further reduced when compared to SELDI as not all proteins may have been transferred at the point of incubation with sera and the capacity for error is much greater. Boyle *et al.*, (2001) demonstrated that 0.75 ng of protein were detectable using SELDI compared to 5 μg of protein by western blotting. The sensitivity of SELDI allows detection of protein samples without pre-concentration, which decreases the time spent on an assay. They suggest SELDI is more sensitive, more rapid and more economic than methods involving SDS-PAGE and western blotting.

4.3.3. Proteins Identified by SELDI.

A number of the proteins putatively identified by corresponding in molecular weight to the proteins cross-reactive with *N. lactamica* antiserum, have been discussed by other authors, including pathogenicity proteins discussed by Tettelin *et al.*, (2000). Prior to publication of the complete genome sequence of *N. meningitidis*, TbpB, bacterioferritin B, pilin, LtgR, PorB, SodC and LtgE were known pathogenicity proteins, however, LpxA and FabZ have subsequently been added to the list.

Pilin, H8 and SodC, with molecular weights of 17.298 16.885 and 17.360 kDa respectively, were suggestions for the 17.378 kDa protein cross-reacting with *N. lactamica* antiserum. Neisserial pili aid adherence of meningococci (and gonococci) to human endothelia (Dunn *et al.*, 1995). Non-piliated strains do not adhere to the cells and cellular damage, caused by LOS, correlates to the level of pilus-facilitated adherance. Pili bind to CD40 receptors on human cells and contact between this receptor and pili cause internalisation of the bacterium (Kallstrom *et al.*, 1997). Perrin *et al.*, (1999) suggested that *N. lactamica* do not possess the genes for pilin homologous to meningococcal pilin genes. However, pili have been observed on commensal *Neisseria* by electron microscopy (Wistreich *et al.*, 1971). Additionally, Aho *et al.*, (2000) showed that all commensal and pathogenic *Neisseriae* that colonise humans posses homologous pilin genes by PCR using primers directed at the conserved regions of meningococcal and gonococcal pilin genes.

The H8 antigen is common to pathogenic *Neisseria*. Black *et al.*, (1985) showed that it was immunogenic and detected antibody responses to it in human meningococcal disease sera. Woods *et al.*, (1987) showed that the monoclonal antibody to H8 did not confer passive protection to mice against meningococcal challenge. However they suggested that active immunisation may be a better method for analysis of H8 as a vaccine antigen. Cannon *et al.*, (1984) found that H8 was present only in pathogenic

Neisseria species. However Aho *et al.*, (1987) showed that as well as having the gene for H8, shown by DNA hybridisation assays, commensal strains also expressed H8.

Meningococcal SodC catalyses the conversion of the superoxide radical anion to hydrogen peroxide preventing production of free radicals which are toxic to the bacterium. Wilks *et al.*, (1998) showed by Southern blotting that commensal *Neisseria* strains, including *N. lactamica*, do not possess SodC.

H8 is not within 2% of the molecular weight of the protein bound by *N. lactamica* antiserum and is a less likely candidate putative protein than the membrane bound proteins which are within 2% of the molecular weight. Therefore, it seems most likely that the protein bound by the *N. lactamica* antiserum is either disulphide bond formation protein B, Pilin or SodC. However, SodC is not present in commensal *Neisseria* (Wilks *et al.*, 1998) so it is unlikely that the protein bound to *N. lactamica* antiserum is SodC.

TonB was listed as one of the putative proteins with a molecular weight within 5% of the molecular weight of the 28.173 kDa protein cross-reactive with the *N. lactamica* antiserum. TonB is involved in iron acquisition by *N. meningitidis* and thought to be an energy transducing protein, spanning the inner membrane and periplasm. It may facilitate uptake of ligands by the TonB-dependent outer membrane porin family of proteins. TonB mutant strains are unable to use haemoglobin, transferrin or lactoferrin as iron sources in culture (Stojiljkovic *et al.*, 1997). However, Desai *et al.*, (2000) showed that pathogenic *Neisseria* can grow, albeit at a low frequency, and therefore utilise exogenous iron without TonB in culture. Recently, Larson *et al.*, (2002) showed that TonB mutant strains were unable to grow within an intracellular location without iron supplementation and they suggested that TonB is required for acquisition of intracellular iron.

PorB has a molecular weight of 33.786 kDa and its molecular weight is within 2% of the molecular weight of the 33.719 kDa protein cross-reactive with *N. lactamica* antiserum. *N. lactamica* do not possess OMPs that are antigenically similar to meningococcal class 2 or 3 proteins when tested for using subtyping antibodes (Kim *et al.*, 1989). However, *N. lactamica* does posses a porin that is more similar to the meningococcal PorB than PorA (Perrin *et al.*, 1999). Of the putative proteins listed, PorB is the only transmembrane protein within 2% of the molecular weight of the bound protein so it is likely that the cross-reactive protein is PorB and that *N. lactamica* posesses a protein antigenically similar to PorB. Although subtyping antibodies are not cross-reactive with the *N. lactamica* porin, these antibodies are raised against the variable regions of the meningococcal PorB proteins suggesting that the *N. lactamica* porin may be antigenically similar in the non-variable regions of the meningococcal PorB. However, human T-cell responses towards the PorB component of the Norwegian OMV vaccine are low in comparison to the responses to the PorA protein (Meyer Naess *et al.*, 1998). Saukkonen *et al.*, (1989) also found that monoclonal antibodies against PorB were not protective against meningococcal infection in rats. Nevertheless, patients surviving systemic meningococcal disease have higher levels of anti-PorB IgG than anti-PorA IgG. PorB conjugate vaccines have also been shown to elicit high levels of bactericidal antibodies.

TbpB was listed as a putative protein with a molecular weight similar to the 66.665 kDa protein cross-reactive with *N. lactamica* antiserum. It has been suggested by a number of authors as a meningococcal vaccine candidate (Ala'Aldeen *et al.*, 1994, Lehmann *et al.*, 1999). Using a mutant strain of *N. meningitidis*, it was shown that the 66.665 kDa cross-reactive protein was TbpB, as a protein of this molecular weight was bound by the serum from the wt strain but not form the mutant strain.

4.4. Conclusions.

Using SELDI to identify the constituent proteins from complex mixtures that cross-react with *N. lactamica* antisera, it has been shown that meningococcal proteins do cross-react with sera raised against protective protein pools of *N. lactamica* and that proteins with homologous immunogenic epitopes to these exist in *N lactamica*. However, using this technique, only information relating to the molecular mass of the cross-reacting proteins could be obtained. The identity of the proteins could be confirmed using further purification steps such as 2D electrophoresis and tryptic digestion of isolated proteins followed by mass spectrometry and molecular weight comparison to predicted peptides. However, this procedure is complicated, labour intensive and time consuming. The method described in this chapter could be used as a rapid method to provide preliminary identification of cross-reactive proteins by mass and proteins of interest could be investigated in further detail.

Chapter 5

Construction and Screening of a *Neisseria lactamica* Genomic Library

5.1. Introduction.

This study has demonstrated that *N. lactamica* OMPs protect mice against lethal meningococcal challenge (Chapter 3). In chapter 4, meningococcal OMPs recognised by antibodies in sera raised against protective *N. lactamica* OMP pools were analysed by SELDI. In this chapter, an alternative approach was used to identify immunogenic *N. lactamica* OMPs using the same sera raised against *N. lactamica* OMPs. An *N. lactamica* genomic expression library was prepared in phage λ, screened for antigen expression with *N. lactamica* antiserum and positive genomic inserts were sequenced. The sequences identified were all homologous to published meningococcal DNA sequences of *N. meningitidis* serogroup B, strain MC58, and serogroup A, strain Z2491. The *N. lactamica* genome has not yet been sequenced. The sequences described in this chapter demonstrate the level of conservation between *Neisseriae* and the cross-reactivity between species.

5.1.1. Genomic Libraries.

A genomic library is a set of clones that are used to represent the entire genome of an organism. Ideally it should comprise of the entire genome of the organism as a set of overlapping cloned fragments of DNA with no misrepresentation of sequences (Nicholl, 2000). The cloned DNA fragments should be large enough to contain whole genes and their flanking sequences (Kaiser and Murray, 1985). Phage λ and cosmid vectors are the best and most commonly used vectors for genomic library production. The size of the genome and the insert size of a library influence the number of clones required to ensure coverage of the genome at 99% confidence (P). The number of clones required (N) can be calculated as follows:

$$N = \ln(1-P) / \ln[1-(x/y)]$$

Where x is the average size of the DNA fragment in base pairs (b.p) and y is the size of the target genome (Ausubel *et al.*, 1995). Sufficiently random cleavage can be obtained

using partial digestions with restriction enzymes. However fragments larger than the capacity of the vector are excluded from the library so enzymes that cut the DNA of interest frequently and without bias are used. *MboI* is a commonly used restriction enzyme with a four nucleotide restriction site and cuts on average one every 256 b.p; thus a collection of fragments that are essentially random and follow a normalised distribution are produced (Nicholl, 2000).

5.1.2. The λ ZAP Vector.

In this study, the λ ZAP vector (Stratagene) was used to create a *N. lactamica* genomic library. It can accommodate DNA inserts of up to 12 kb in length. The pBK-CMV plasmid vector containing inserted DNA can be excised from the λ ZAP vector allowing characterisation of the insert in a plasmid system. Cells containing λ ZAP can express the insert as a fusion protein and can be screened for expression using antibody probes. Expression of the insert is driven by the *lacZ* promotor and induced by IPTG and cells containing phage with inserted DNA are selected for by blue/white colour screening. T3 and T7 promotors flank the multiple cloning site of the plasmid vector and contain common primer sites for DNA sequencing. These promotors are also recognised by DNA dependent RNA polymerase, which initiate the synthesis of RNA from double stranded DNA templates. Vectors carrying the bacteriophage T3 and T7 promotors can be used to express cloned genes *in vivo*. The plasmid contains the f1 origin of replication from the M13 bacteriophage and this is used for excision of the cloned DNA in plasmid form (Figure 5.1). When cells containing λ ZAP are co-infected with helper phage (M13), single stranded DNA copies of the cloned DNA and pBK-CMV DNA are produced. This is circularised by the gene II product of the helper phage and secreted from the host into the medium. Treatment of the cells and λ ZAP at 70°C causes cell and phage lysis but the single stranded copy of the pBK-CMV plasmid containing cloned DNA is not affected. *E. coli* cells are then infected with the single

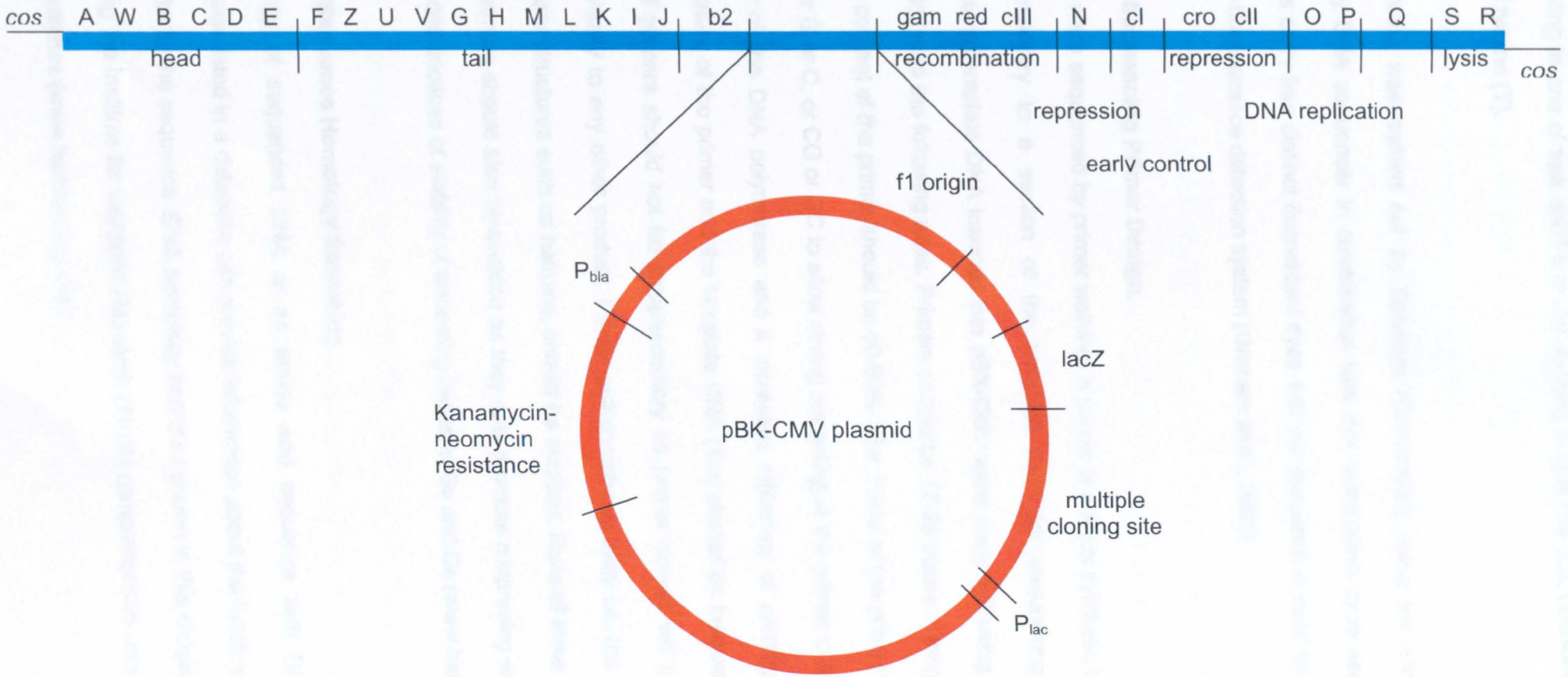

Figure 5.1 Map of the λ ZAP Express vector and the pBK-CMV plasmid encoded within it. Exogenous DNA is inserted in the multiple cloning site.

stranded plasmid which is converted to a double stranded plasmid and maintained within the cells (www.stratgene.com, www.csun.edu).

5.1.3. Immunological Screening of a Genomic Library.

The *N. lactamica* genomic library was screened with *N. lactamica* antiserum to identify fusion proteins expressed by λ ZAP that were recognised by antibodies in the serum. Once identified, these inserts were excised from λ ZAP and maintained as inserts in pBK-CMV as described in the previous section. Immunological screening of λ ZAP is in principle similar to western blotting. Due to the lytic capabilities of the λ ZAP vector, *E. coli* cells infected with the phage are lysed and plaque formation is observed on a lawn of cells. Plaques are transferred to nitrocellulose membranes and these are screened using serum and cross-reactive plaques identified and isolated. These plaques are then used to infect fresh cells and pBK-CMV plasmids containing cross-reactive inserts are produced. Sequencing of the inserts can be carried out using the T3 and T7 sequencing primers from the sites flanking the inserted DNA.

5.1.4. DNA Sequencing.

Sequencing a gene provides useful information about coding regions of genes, homology of the genes and non-coding regulatory regions of the gene. Sequencing techniques are based on electrophoretic techniques and were developed by Allan Maxam and Walter Gilbert (Maxam *et al.*, 1977) and Fred Sanger and Alan Coulson (Sanger *et al.*, 1977) respectively. The Maxam-Gilbert method uses chemicals to cleave radiolabelled DNA and generate a set of oligonucleotides differing by one nucleotide (Nicholl, 1994). The Sanger-Coulson method utilises *E. coli* DNA polymerase large fragment (Klenow fragment) to synthesize a complementary copy of a single stranded DNA template from a primer. DNA polymerase is capable of using dideoxynucleoside triphosphates (ddNTP) as substrates. When a ddNTP is incorporated to the 3' end of the growing DNA strand, elongation is terminated, generating set of fragments

terminating in one of four dNTPs corresponding to guanine (G), cytosone (C), adenine (A) and tymine (T).

Sequencing was carried out by Cytomyx (Cambridge) using the MegaBace 1000 capillary DNA sequencer in combination with dye termination cycle sequencing. The ddNTPs carry four distinct fluorescent dyes and the sequence is read as a colour code using a fluorescence detection system (Graham *et al.*, 2001)

5.1.5. Sequencing Primer Design.

Clones were sequenced by primer walking. A primer is a short synthetic oligonucleotide complementary to a section of the template DNA for sequencing. Primers for sequencing template DNA inserted into pBK-CMV were designed using Primer Select (DNAStar) and the following rules. Primers should be 17-28 bases in length. The G and C base content of the primer should be 50-60%. The 3' end of the primer should consist of either G or C, or CG or GC to allow strong annealing of the primer to the template for binding of the DNA polymerase and it increases efficiency of priming. The melting temperature of the primer and the template DNA (Tm) should be between 55-80°C. 3'-ends of primers should not be complementary as primer dimers will be synthesised preferentially to any other product. Primer self-complementarity i.e. the ability to form secondary structures such as hairpins, should be avoided. Runs of three or more Cs or Gs in primers should also be avoided as they may promote mispriming with G or C-rich sequences because of stability of annealing between Gs and Cs (www.bioquest.org).

5.1.6. Sequence Homology Searching.

Homology of sequenced DNA or an amino acid sequence with DNA or protein sequences listed in a database can provide information about the function of the protein coded for by the sequence. DNA homology searches shown in this chapter were carried out using the Institute for Genomic Research (TIGR) comprehensive microbial resource blast searches (www.tigrblast.tigr.org).

5.1.7. *N. meningitidis* Genome Sequences.

The complete genome sequence of *N. meningitidis*, serogroup B, strain MC58 has been determined by Chiron and of serogroup A, strain Z2491 by the Sanger Center (Tettelin *et al.*, 2000, Parkhill *et al.*, 2000). Sequencing of a serogroup C meningococcal strain (FAM18) by the Sanger Center is in progress (www.sanger.ac.uk). *N. meningitidis*, strain MC58 has a genome of 2,272,351 bp and an average G and C content of 51.5%. The 2158 open reading frames (ORF) were identified coding for 678 proteins. Of the 2158 ORFs, 91.2% have homology to ORFs in Z2491 and the ORFs without similarity are hypothetical proteins. The genome of Z2491 is 2,184,406 bp in length with a G and C content of 51.8% and it contains 2121 ORFs. Both the genome of MC58 and Z2491 contain a number of repetitive elements providing the ability of the genes to undergo phase variation and contributing to the ability of *N. meningitidis* to evade the immune system. These could encourage sequence variation by acting as the target sites for recombination and the production of new alleles of the gene.

5.2. Results.

5.2.1. *N. lactamica* Genomic Library.

Genomic DNA from *N. lactamica* strain Y92-1009, was prepared using a CsCl gradient and partially digested with *MboI*. Digested DNA of between 1 and 4 kb was gel purified and used to produce the genomic library. Digested DNA was ligated and packaged in to the λ ZAP vector according to the manufacturer's instructions. The number of recombinant phage required to cover the entire genome of *N. lactamica* was determined to be 4050 clones (P=0.01). This was calculated on the assumption that the genome of *N. lactamica* is similar in length to that of *N. meningitidis* and based on a working dilution of 250 phage per plate. Approximately this number of recombinant phage were used to transfect *E. coli*, strain XL1-Blue MRF', and the phage from the plaques produced were recovered in SM buffer to form the genomic library. The library was screened for recombinant phage that cross-reacted with serum raised in rabbits against

N. lactamica OMPs. Initially, the screening was with serum raised against *N. lactamica* low molecular weight (LMW) OMPs (43 kDa), but no cross-reactivity at all was observed. However, cross-reactivity of the phage library was observed on screening with serum raised against *N. lactamica* detergent extract (DE) OMPs. Cross-reactive phage were identified and re-screened to eliminate false positive reactions. The pBK-CMV vector was excised from the rescreened cross-reactive phage with the help of ExAssist helper phage and the plasmids were able to replicate in the *E. coli*, strain XLOLR. Recombinant plasmids were sequenced with the T3 and T7 primers whose annealing sites flank the insert (Figure 5.2). As sequencing only yields approximately 500 bases of reliable sequence and the *N. lactamica* inserts were all between 1 and 4 kb in length, further sequencing was required. This was carried out by primer walking i.e. primers were designed to anneal to the ends of the previous sequences and sequencing of the inserts was complete when sequence data from the T3 end of the insert overlapped with sequence data from the T7 end of the insert. Table 5.1 shows a list of the clones sequenced and the meningococcal proteins to which they show homology using the TIGR website to carry out 'Blast' searches (www.tigr.org/cmr-blast/). Although a number of different primers were designed, the sequence of clone 4 remains incomplete due to inability to obtain overlapping sequence data. Homology of the *N. lactamica* sequences with meningococcal genes is high and each of the inserts shows homology to more than one gene which it overlaps. Complete sequence data for each of the *N. lactamica* inserts is shown on appendix A3. Overlap with the vector is highlighted.

5.2.2. Alignment of *N. lactamica* DNA Sequences with Homologous Meningococcal Genes.

The sequences of meningococcal proteins with homology to the *N. lactamica* sequences were obtained from the NCBI database and searching GenBank (www.ncbi.nlm.nih.gov). Homology of the *N. lactamica* sequences with these genes is shown in appendix A4.

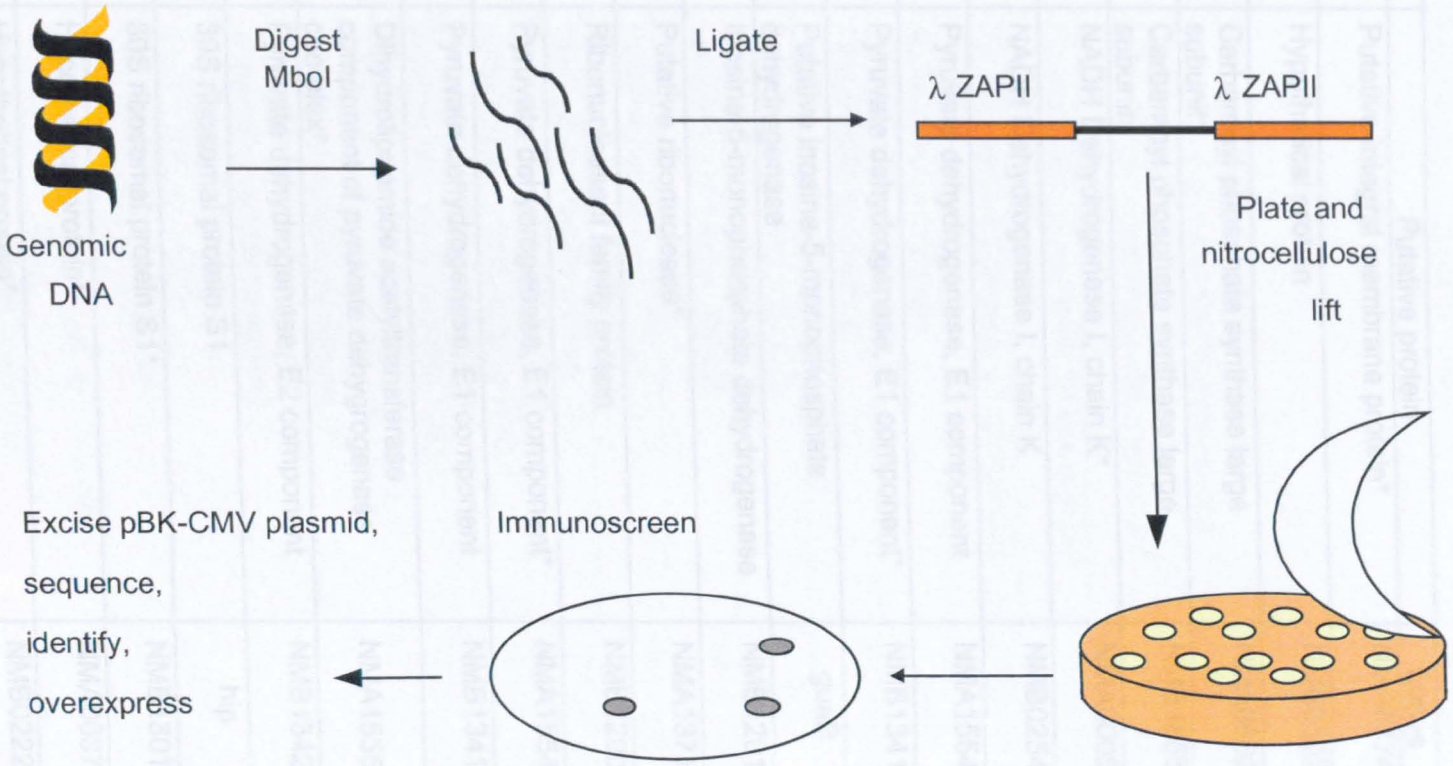

Figure 5.2 Schematic showing Production Processes of a *N. lactamica* Genomic Library. *N. lactamica* genomic DNA was partially digested with Mbo I, ligated and packaged into λZAP phage which were used to transfect *E. coli*. Cross-reactive recombinant phage were screened for with *N. lactamica* antiserum. The pBK-CMV plasmid was excised from the recombinant phage and *N. lactamica* inserts sequenced.

Table 5.1 Putative Identification of *N. lactamica* sequenced inserts and their Homology to meningococcal sequences. (* proteins whose coding region was also found with translation of the DNA and are in the same orientation as the promotor)

Clone	Putative protein	Locus	Serogroup	Homology
3	Putative integral membrane protein*	NMA0174	A	89% (496/552)
	Hypothetical protein	NMB0102	B	89% (466/518)
4	Carbamoyl phosphate synthase large subunit*	NMA0602	A	97% (340/347)
	Carbamoyl phosphate synthase large subunit	NMB1855	B	94% (330/348)
	NADH Dehydrogenase I, chain K*	NMA0005	A	95% (291/304)
	NADH Dehydrogenase I, chain K	NMB0254	B	95% (290/304)
	Pyruvate dehydrogenase, E1 component	NMA1554	A	93% (653/696)
	Pyruvate dehydrogenase, E1 component*	NMB1341	B	93% (653/696)
8	Putative inosine-5-monophosphate dehydrogenase	guaB	A	96% (667/691)
	Inosine-5-monophosphate dehydrogenase	NMB1201	B	95% (657/691)
	Putative ribonuclease*	NMA1371	A	85% (722/847)
	Ribonuclease II family protein	NMB1200	B	85% (720/847)
9	Pyruvate dehydrogenase, E1 component*	NMA1554	A	94% (646/684)
	Pyruvate dehydrogenase, E1 component	NMB1341	B	94% (646/684)
	Dihydrolipoamide acetyltransferase component of pyruvate dehygrogenase complex*	NMA1555	A	84% (909/1074)
	Pyruvate dehydrogenase, E2 component	NMB1342	B	85% (903/1060)
10	30S ribosomal protein S1	hip	A	90% (1114/1233)
	30S ribosomal protein S1*	NMB1301	B	90% (1114/1233)
19	Hypothetical protein*	NMA0037	A	90% (429/475)
	Hypothetical protein*	NMB0222	B	90% (429/475)
	Hypothetical protein*	NMA0038	A	90% (176/195)
	Hypothetical protein*	NMA0036	A	94% (270/285)
20	Putative integral membrane protein	NMA1899	A	83% (780/936)

(Table 5.1 continued)

Clone	Putative protein	Locus	Serogroup	Homology
	Hypothetical protein*	NMB1645	B	84% (786/936)
	Putative integral membrane protein*	NMA1898	A	83% (698/834)
	Hypothetical protein	NMB1644	B	83% (702/834)
23	Dihydrolipoamide acetyltransferase component of pyruvate dehygrogenase complex*	NMA1555	A	89% (1152/1292)
	Pyruvate dehydrogenase, E2 component	NMB1342	B	89% (1153/1291)
	Pyruvate dehydrogenase, E1 component*	NMA1554	A	91% (632/693)
	Pyruvate dehydrogenase, E1 component	NMB1341	B	91% (632/693)
25	Alanyl-tRNA synthetase	alaS	A	94% (2277/2420)
	Alanyl-tRNA synthetase*	NMB1595	B	93% (2270/2420)
	Lactoferrin binding protein B	lbpB	A	86% (543/626)
	Lactoferrin binding protein B	NMB1541	B	85% (469/546)
26	Putative hydrolase*	NMA0387	A	90% (276/304)
	Glyoxylase II family protein	NMB2049	B	91% (277/304)
	putative Na(+)-translocating NADH-ubiquinone reductase subunit C	NMA0750	A	93% (669/716)
	putative Na(+)-translocating NADH-ubiquinone reductase subunit C	NMB0567	B	92% (660/716)
	putative Na(+)-translocating NADH-ubiquinone reductase subunit D*	nqrD	A	97% (400/411)
	putative Na(+)-translocating NADH-ubiquinone reductase subunit D	NMB0566	B	88% (445/501)
	Putative periplasmic protein		A	85% (207/243)
	Conserved hypothetical protein	NMB2050	B	81% (187/229)
34	Putative thiamine biosynthesis protein*	NMA0746	A	94% (616/651)
	Thiamine biosynthesis lipoprotein	NMB0563	B	91% (657/717)
	Na(+)-translocating NADH-ubiquinone reductase subunit E		A	91% (545/598)
	Na(+)-translocating NADH-ubiquinone reductase subunit E*	NMB0565	B	91% (547/598)

(Table 5.1 continued)

Clone	Putative protein	Locus	Serogroup	Homology
36	Haemagglutinin / haemolysin-related protein*	NMB1768	B	89% (1705/1909)
42	DprA homologue	dprA	A	91% (1084/1182)
	DNA processing chain A*	NMB0116	B	90% (1090/1204)
	Nitrogen assimilation regulatory protein*	NMB0115	B	84% (827/982)
	Putative two-component transcriptional regulator		A	84% (825/982)
	Hypothetical protein*	NMA0157	A	84% (381/453)
	Putative smg protein	NMB0117	B	83% (378/453)
53	BirA bifunctional protein	birA	A	85% (879/1027)
	BirA protein / Bvg accessory factor*	NMB2075	B	85% (880/1028)
	Putative periplasmic protein		A	91% (390/425)
	Hypothetical protein	NMB2074	B	90% (243/270)
59	Pyruvate dehydrogenase, E2 component	NMB1342	B	83% (637/765)
	Dihydrolipoamide acetyltransferase component of pyruvate dehygrogenase complex*	NMA1555	A	83% (637/765)
	Pyruvate dehydrogenase, E1 component	NMB1341	B	94% (643/681)
	Pyruvate dehydrogenase, E1 component*	NMA1554	A	94% (643/681)
63	Putative periplasmic protein	NMA0386	A	89% (370/412)
	Conserved hypothetical protein*	NMB2050	B	90% (373/410)
	Ubiquinol cytochrome c reductase, cytochrome c1	NMB2051	B	86% (302/348)
	Cytochrome c1*	NMB0385	A	86% (302/348)
73	HemK protein*	NMA0369	A	93% (663/706)
	HemK protein	NMB2065	B	91% (700/761)
	Putative amino acid permease ATP-binding protein		A	64% (477/735)
	Amino acid permease ATP-binding protein	NMB0696	B	64% (481/743)

(Table 5.1 continued)

Clone	Putative protein	Locus	Serogroup	Homology
75	Probable ATP-dependent DNA helicase	dinG	A	90% (1031/1139)
	Probable ATP-dependent DNA helicase*	NMB0287	B	90% (1040/1149)
	Hypothetical inner membrane protein		A	96% (225/234)
	Hypothetical protein	NMB0288	B	96% (217/225)

5.2.3. Translation and Alignment of *N. lactamica* Sequences with Meningococcal Protein Sequences.

N. lactamica sequences were then translated into their amino acid sequences in all six reading frames, three forward and three reverse, using Expasy-Translate tool (www.expasy.ch/tools/dna.html). Each of the translated amino acid sequences were compared to the amino acid sequences of the meningococcal proteins with which the DNA sequences had homology and the DNA coding regions found. The putative proteins coded for by the *N. lactamica* inserts are indicated by a star on Table 5.1 and the proteins coding region of the sequenced inserts are indicated on appendix A3. Each of the *N. lactamica* inserts codes for at least one protein but often the sequence for each protein is incomplete. The coding regions are in the same reading frame as the promotor.

5.2.4. Specificity of the Immunological Recognition of Phage Proteins.

ELISA was used to assess the cross-reactivity of recombinant phage with a variety of human and animal meningococcal and *N. lactamica* antisera. Antisera against anthrax, pneumococcal and TB cell lysates were also used as negative control sera. Empty phage were used as a control for cross-reactivity of the sera with phage rather than the inserts. The negative control sera showed no cross-reactivity with the recombinant phage and the meningococcal sera only showed reactivity with recombinant phage. However, the sera were broadly cross-reactive with each of the recombinant phage (Table 5.2). The reactivity of human convalescent sera with the recombinant phage was also assessed (Table 5.3) and the results were variable with some patient sera being broadly cross-reactive with the recombinant phage and others being reactive with only a few recombinant phage.

5.3. Discussion.

A number of authors have described the use of genomic libraries prepared from *N. meningitidis* to identify genes of interest. Frosch *et al.*, (1989) isolated the genes

Clone	Antiserum													
	TB	Anthrax	S.pn	N I DE	N.c OMV	K454 OMV	MC58 wt OMV	MC58 cap-OMV	N.I LMW	N.I MMW	N.I HMW	N.I L1	N.I L2	N.I L3
3	<100	<100	<100	1924	917	1806	231	457	1469	1227	500	6786	789	1207
4	<100	<100	<100	3542	785	5135	608	501	595	302	509	1245	564	691
8	<100	<100	<100	753	486	388	166	296	161	236	653	<100	396	432
9	<100	<100	<100	191	341	487	432	230	1809	<100	695	<100	430	339
10	<100	<100	<100	8068	584	604	4939	261	810	1432	1403	1882	932	1341
19	<100	<100	<100	454	419	614	381	300	441	172	485	168	950	381
20	<100	<100	<100	2147	625	572	326	446	689	206	702	532	609	449
23	<100	<100	<100	234	420	343	<100	805	367	<100	178	<100	440	<100
25	<100	<100	<100	1385	631	408	142	283	1189	890	723	1086	621	533
26	<100	<100	<100	249	271	273	<100	386	128	<100	<100	<100	455	286
34	<100	<100	<100	1456	474	582	424	453	195	981	1425	1373	833	953
36	<100	<100	<100	3362	501	578	298	371	1538	2098	2227	2322	1109	1249
42	<100	<100	<100	1092	443	518	227	243	3514	1875	662	672	645	733
53	<100	<100	<100	4398	592	660	393	378	3339	1018	880	1733	530	883
59	<100	<100	<100	344	321	652	661	557	131	<100	<100	<100	357	239
63	<100	<100	<100	3921	758	648	482	1003	2260	2175	1991	2735	1085	1656
73	<100	<100	<100	476	332	380	498	144	508	447	2102	<100	781	521
75	<100	<100	<100	295	377	464	304	358	6738	196	1578	<100	917	478
empty	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100

Table 5.2 Raw titres for cross-reactivity of animal sera with recombinant phage. Anthrax, TB and pneumococcal antisera were used as negative control sera and empty phage particles as negative control phage.

Clone	26	27	PV	TE	251618	252322
3	1116	1255	5970	515	<100	2563
4	1179	1082	1492	<100	<100	323
8	333	819	1087	<100	186	648
9	776	510	528	<100	<100	<100
10	750	1218	1530	<100	<100	370
19	972	1262	938	<100	<100	362
20	494	1184	1310	287	277	559
23	182	667	244	232	<100	246
25	230	651	455	144	<100	425
26	<100	635	141	<100	<100	358
34	263	774	430	168	<100	388
36	130	717	956	<100	378	776
42	197	618	383	195	<100	549
53	222	691	812	<100	289	702
59	260	344	129	142	<100	<100
63	557	808	1896	<100	226	612
73	469	653	926	<100	<100	505
75	660	772	1680	<100	<100	434
empty	<100	<100	<100	<100	<100	<100

Table 5.3 Raw titres for cross-reactivity of human convalescent sera with recombinant phage. Human sera shows variable reactivity with the inserts and inconsistency between sera.

responsible for capsule production in *N. meningitidis* serogroup B. A cosmid library in *E. coli* was screened with a monoclonal antibody specific for capsular polysaccharide of group B meningococci. A 24 kb genomic fragment elicited detectable capsular polysaccharide in *E. coli* and was made up of five regions all necessary for capsule synthesis. A genomic library of *N. meningitidis* in λ ZAP II was screened for DNA encoding TbpA (Palmer *et al.*, 1993). Using this library, *tbpA* was isolated with a DNA probe and shown to be highly homologous to that of *N. gonorrhoea.* Using a similar method, Ait-Tahar *et al.*, (2000) screened a meningococcal genomic library for cross-reaction with human convalescent sera. The most promising positive clone, *autA*, was characterised. It encoded the putative autotransporter A protein of *N. meningitidis*, having a peptide sequence sharing molecular characteristics of the autotransporter family of proteins, and is highly conserved among meningococci and for this reason they suggested it as a potential vaccine candidate due to being highly conserved.

Zysk *et al.*, (2000) described the use of a pneumococcal genomic plasmid expression library to identify unknown immunogenic proteins. Recombinants were screened with a convalescent phase serum and 78 cross-reactive proteins were identified and sequenced. With a similar aim and methodology, the *N. lactamica* genomic library described in this chapter was used to identify immunogenic *N. lactamica* proteins.

A plasmid-based genomic library of *N. lactamica* has been previously prepared (Morgan *et al.*, 1996) to clone the *NlaIII* restriction modification system and express it in *E. coli*. *NlaIII* is a type II restriction endonuclease. However, to my knowledge, a *N. lactamica* genomic expression library has not been screened to assess the presence of immunogenic sequences.

For this study, a λ ZAP phage display library was prepared from *N. lactamica* genomic DNA and various *N. lactamica* genes were identified as coding for proteins that were immunogenic and could be involved in protection. These proteins have been shown by

ELISA to cross-react with a variety of animal and human sera raised against meningococci.

5.3.1. Immunogenic *N. lactamica* Proteins.

A variety of *N. lactamica* proteins were immunogenic as detected by cross-reactivity with *N. lactamica* antiserum and recombinant phage ELISA. Eighteen recombinant phage were cross-reactive and these were sequenced to identify the encoded genes. Sequencing showed that 7 clones were identical and one insert could not be completely sequenced. The remaining clones showed a high degree of homology to a variety of meningococcal serogroup A and B genes. These sequences coded completely or partially for a total of 23 proteins.

The putative proteins identified by sequence comparison with *N. meningitidis* are described on Table 5.1 of this study. Of these, lactoferrin binding protein B (LbpB), encoded for by clone 25, and haemagglutinin/haemolysin-related protein, encoded for by clone 8, are putative pathogenicity proteins (Tettelin *et al.*, 2000). LbpB has been discussed by a number of authors as a potential vaccine candidate. It binds lactoferrin, an alternative iron source, and is expressed in conjunction with LbpA under conditions of iron stress. Meningococcal LbpB shows homology to meningococcal TbpB, and the gene encodes a protein of 77.5 kDa, with 33% identity to TbpB. This homology consists of two stretches of negatively charged residues which might be involved in lactoferrin binding. Mutants lacking LbpB exhibit a reduced ability to bind lactoferrin (Petterson *et al.*, 1998). Petterson *et al.*, (1999) examined sequence conservation of LbpB in a variety of meningococcal strains. Sequence variability was high, especially among the negatively charged residues, so only the charge of these residues is probably important for lactoferrin binding. The variability of the sequences was suggested to be a drawback for vaccine development purposes. Translation of clone 25 showed that the section of the sequence with homology to LbpB was in the opposite direction to the promotor and it could not therefore be expressed.

A genomic library prepared from *N. meningitidis* in M13 phage was used by Newcombe *et al.*, (2000) and screened for proteins present in convalescent sera but not in acute sera. Using this method they identified a number of outer membrane proteins, virulence proteins and regulatory proteins. The proteins identified are listed on Table 5.4 (Dr J. Newcombe, personal communication). Alanyl tRNA synthetase, encoded for by clone 25, was identified both by this work and the method described in this chapter. A haemagglutinin precursor and a haemolysin activator protein were also identified and a haemagglutinin/haemolysin related protein, coded for by clone 36, was identified using the *N. lactamica* genomic library. The M13 phage display library was also screened with the same *N. lactamica* antiserum used for screening in this chapter. The only protein identified was NusG. This protein was not identified by screening the *N. lactamica* genomic library in this chapter.

The remaining sequences identified, are not described as encoding for putative pathogenicity proteins (Tettelin *et al.*, 2000). However, CarB, encoded for by clone 4, and dihydrolipoamyl transferase, coded for by clones 59, 23 and 9, have been mentioned by other authors (Lawson *et al.*, 1995, Ala Aldeen *et al.*, 1996). The carbamoyl phosphate synthase enzyme is coded for by the genes *carB* and *carA* which catalyses the formation of carbamoyl-phosphate from carbon-dioxide, adenosine-triphosphate and glutamine and is the first step in the arginine and pyrimidine biosynthetic pathways. It has primarily been investigated in *N. gonorrhoea* because 20-30% of naturally occurring clinical isolates have absence of in the carbamoyl phosphate synthase enzyme (Lawson *et al.*, 1995). The genes are co-transcribed in the order *carA-carB* and they are separated by a large intervening sequence ranging in size from approximately 2.2 to 3.7 kb. If the absence of carbamoyl phosphate synthase, observed in gonococci, is also observed in meningococcal clinical isolates, this protein is probably not suitable as a vaccine candidate.

Putative protein
Porin
Solvent tolerance protein
Virulence associated protein
Pilin
Sensor histidine kinase
Dihydro-deoxyphosphooctonate aldolase
Shikimate dehydrogenase
Haemagglutinin precursor
Haemolysin activator precursor
Transmembrane transport protein
ATP-dependent helicase
Thiamine biosynthesis protein
Thiol-disulphide interchange protein
M5c methyltransferase
Alanyl t-RNA sythetase
IPP transferase
Proylyl endopeptidase
Hypothetical proteins

Table 5.4 Proteins identified by screening a *N. meningitidis* genomic library with human conavlescent serum. (Infomation supplied by J. Newcombe)

Ala'Aldeen *et al.*, (1996) screened a λ ZAP II expression library with rabbit serum raised against 70 kDa proteins purified from OMVs of *N. meningitidis* and identified dihydrolipoamide acetyltransferase, encoded for by the gene *aceF*, as one of the reactive clones. It is a component of the pyruvate dehydrogenase complex. It is highly conserved across both prokaryotic and eukaryotic species and is immunogenic in animals when used as purified protein or as a component of live meningococci. Convalescent patient sera are also cross-reactive with dihydrolipoamide acetyltransferase. However, it is unknown whether the reactivity of human sera is with conserved epitopes of the proteins and shared with man and therefore whether use of this protein as a vaccine antigen would risk tolerance or autoimmunity. Normal human sera were also cross-reactive with the protein but meningococci are commensals of the nasopharynx and this carriage may elicit antibody responses to bacterial dihydrolipoamide acetyltransferase.

All of the *N. lactamica* sequences identified in this chapter consisted of genes encoding proteins that are cross-reactive with sera raised against *N. lactamica* OMPs as shown by ELISA. Therefore all of the proteins, either individually or pooled may be involved in the protection elicited by *N. lactamica* shown in chapter 3. Most of the *N. lactamica* sequences from cross-reactive recombinant phage consisted of more than one gene and encoded for more than one protein. It is possible that all or some of the proteins encoded for are immunogenic or protective if expressed and in the correct orientation. Production of recombinant proteins would provide further information about which are the immunogenic proteins and their protective efficacies. Time did not allow for continuation of this study. It would have been interesting to compare the results of screening the library with both *N. lactamica* and human *N. meningitidis* antiserum. Proteins identified by both screening conditions may be the most promising vaccine candidates. However, at the time of screening, the quantities of human meningococcal antiserum required were not available. The ELISA data shown in this chapter suggests that the selected recombinant phage are cross-reactive with meningococcal antiserum.

Screening with meningococcal antiserum could also be carried out once recombinant proteins have been produced and purified. Screening with sera only identifies immunogenic proteins, indicating that these proteins may be involved in protection, but protective efficacy can only be assessed in an animal model.

5.3.2. Problems Sequencing Clone 4.

Clone 4 proved difficult to completely sequence. Sequence data was obtained from each end of the insert. Homology of each of these with *N. meningitidis* suggests that clone 4 consists of two fragments of DNA from different parts of the *N. lactamica* genome. These have probably become ligated into the same vector at the point of preparation of the genomic library and a 2.5 kb region covering the join between the two sections was impossible to sequence. Sequencing difficulties are often encountered and there are a number of different causes. The quality of the DNA template is very important for sequencing. Contaminants such as salt, carbohydrate, protein, excessive dNTPs or primers can effect the data (www.le.ac.uk). Mixed sequence data can be obtained as a result of two templates being present in the sequencing reaction. However, this was probably not the problem encountered here as the template was used to obtain part of the sequence data. DNA that contains repeat sequence regions is often very difficult to sequence. For example, if a homopolymeric region is encountered, the rate at which the relevant nucleotide is used is greater than the other nucleotides, and the sequencing reaction may stop prematurely. The worst repeat regions tend to be G C based as there is difficulty incorporating the bases or the template DNA can form secondary structures in these regions. The *N. meningitidis* genome contains a number of repetitive elements which are associated with phase variation, more than any other bacterial genome sequenced to date (Tettelin *et al.*, 2000, Parkhill *et al.*, 2000). In *Neisseria* these repeat regions generally consist of G or C repeats and may contribute difficulty in sequencing (Snyder *et al.*, 2001). CarB, encoded for by the partly sequenced clone 4, contains a number of repetitive sequences and these may be responsible for

the problems encountered here and in total CarB has a GC content of 57% (Lawson *et al.*,1995).

5.4. Conclusions.

Using a λ ZAP Express library, 15 different fragments of *N. lactamica* DNA were shown to cross-react with serum raised against protective *N. lactamica* OMPs. One fragment was not completely sequenced, probably due to secondary structure of the DNA caused by repetitive elements, but the remainder were completely sequenced and contained either partial or complete genes encoding for 23 separate proteins. The genes and encoded proteins showed high homology to equivalent meningococcal proteins. The clones are broadly cross-reactive with animal and human sera against meningococci implying that the proteins are immunogenic and that they may be possible vaccine candidates. Further investigations need to be carried out to identify the important proteins for immunogenicity or protective efficacy. This comprises of preparation of recombinant proteins and efficacy trials in animal models. However, the method used in this study has proved effective for the identification of novel vaccine candidates. It may be interesting to re-screen the library for further cross-reactive antigens with either the same serum or variety of meningococcal antiserum. It would also be interesting to discover the homology of the immunogenic proteins identified in this study with other meningococcal strains whose genomes are not yet sequenced using, for example, Southern blotting.

Chapter 6

General Discussion

6.1. Overview.

This study presents the important observation that vaccines based on *N. lactamica* protect mice against lethal meningococcal challenge. To identify the components responsible for this protection, *N. lactamica* detergent extracted OMPs were separated by preparative electrophoresis, into three pools of proteins consisting of LMW, MMW and HMW proteins. The LMW protein pool was shown to be the most protective of these. Further separation of this pool indicated that proteins of 25-43 kDa provided protection and those *N. lactamica* proteins with a molecular weight of greater than 43 kDa were unprotective at meningococcal challenge doses higher than 1×10^8 CFU. This differs from with data reported by Sierra *et al.*, (1990) who supplemented an OMV vaccine with HMW proteins to increase the protective efficacy of the vaccine. *N. lactamica* OMPs were shown to elicit antibodies that were cross-reactive by ELISA with a broad range of meningococci of different serogroups, serotypes and serosubtypes and these cross-reacting antibodies were of isotypes IgG1 and IgG2b. Using western blotting, *N. lactamica* OMP antiserum was most cross-reactive with meningococcal proteins of 30 kDa and 67 kDa. In a western blotting study with human meningococcal disease sera, Wedege *et al.*, (1998) identified a *N. meningitidis*, 30 kDa protein as PorB. In this study, mouse *N. lactamica* OMP antisera were not bactericidal and although rabbit antisera showed some bactericidal activity, pre-immune rabbit serum was also bactericidal to the target strains thus, the titres did not correlated with protection. Bactericidal antibodies are directed towards PorA in OMV vaccines (Van der Ley, 1992, Saukkonen, 1998). *N. lactamica*, unlike meningococci, do not posess a PorA protein (Derrick *et al.*, 1999) which may be responsible for the lack of bactericidal activity of *N. lactamica* anitserum. West *et al.*, (2001) have also shown that a protein antigen can be protective in a mouse disease model without inducing bactericidal antibodies.

SELDI and *N. lactamica* genomic library screening were used to identify immunogenic *N. lactamica* proteins. Meningococcal OMPs cross-reactive with *N. lactamica* antiserum were identified by SELDI. Using the molecular weight of the cross-reacting proteins and

comparisons with the meningococcal protein data base (www.expasy.co.uk), a list of putative proteins cross-reactive with *N. lactamica* antiserum was generated and it was shown that proteins with epitopes homologous to the cross-reacting meningococcal proteins were present in *N. lactamica*. These may provide the protection observed against lethal meningococcal challenge following vaccination with *N. lactamica* OMPs. However, this technique only provided conclusive identification of one protein, the 66.7 kDa cross-reactive meningococcal protein, TbpB. Further conclusive results could have been provided if the meningococcal proteins were further separated into individual proteins by preparative 2D electrophoresis for example, and the proteins cleaved to obtain a set of peptides corresponding to each protein. Comparison of the peptide mass spectra with those of theoretically cleaved proteins can provide the identity of a protein, though there was not sufficient time within this study. This is the first study that has assessed cross-reactivity of complex mixtures of proteins with serum raised against complex mixtures of proteins using SELDI. The majority of meningococcal proteins cross-reacting with *N. lactamica* serum had molecular weights below 43 kDa and *N. lactamica* OMPs less than 43 kDa provided protection to mice against lethal meningococcal challenge.

Using a *N. lactamica* genomic library, the DNA sequences of recombinant *N. lactamica* proteins cross-reactive with *N. lactamica* antiserum were obtained. Comparison with the complete genome sequences of *N. meningitidis* serogroup A (Parkhill *et al.*, 2000) and *N. meningitidis* serogroup B (Tettelin *et al.*, 2000) using TIGR to carry out blast searches (www.tigr.org) provided a list of meningococcal proteins with homology to the *N. lactamica* sequences. After translation of the DNA sequences into their amino acid sequences, the coding regions of the DNA were obtained and therefore the proteins most likely to be expressed by the *N. lactamica* sequences. Fifteen cross-reactive sequences coded either partially or completely for 23 different proteins.

The lists of proteins identified by SELDI and the genomic library did not overlap. However, these techniques do have the potential to identify corresponding proteins if SELDI had been used to analyse and characterise individual proteins rather than complex mixtures. The genomic library approach is more accurate for identifying cross-reacting proteins and potential vaccine candidates as sequence data was obtained. In this study, SELDI provided information regarding the molecular weight of cross-reacting proteins only, and therefore did not have the sensitivity to provide definitive identification of meningococcal proteins cross-reacting with *N. lactamica* antiserum.

Alternatively, western blotting of 1D and 2D polyacrylamide gels of *N. meningitidis* OMPs could have been used as a method for identification of immunogenic proteins that cross-react with *N. lactamica* antisera. However,this approach is limiting as the sample is not in its native form so not all immunogenic proteins will be identified. Sanchez *et al.*, (2001) used western blotting to identify bactericidal antibodies towards antigens that are common to commensal and pathogenic neisseriae. They suggested analysis of cross-reactive antigens by western blotting precludes detection of antibodies that react with epitopes that are conformational.

Apart from the OMPs identified and discussed in chapters 4 and 5, a number of other proteins have been described by other authors and identification of these may also have been expected by either or both of the methods used in these chapters. For example Cann and Rogers (1989) identified antigens of 70 kDa, 65 kDa and 15-20 kDa common to meningococci and *N. lactamica* that were recognised by antibodies in convalescent sera from children. Troncoso *et al.*, (2000) discussed 32 kDa and 55 kDa proteins common to *N. lactamica* and *N. meningitidis*. However, none of these proteins were identified and their molecular weights were obtained by western blotting, so it cannot be deduced whether some of these proteins were in fact identified by SELDI. For example, the 65 kDa protein identified by Cann and Rogers could be the same as the 66.7 kDa protein identified by SELDI. Also the proteins identified by Troncoso *et al.*, may

correspond to the 33.7 kDa and 53.2 kDa proteins identified as putative proteins by SELDI. Various proteins were identified by Wedege *et al.*, (1998) as immunogenic by western blotting of human post vaccination sera with *N. meningitidis* OMVs. Again, a protein of 65 kDa was identified along with a protein of approximately 30 kDa, identifed as the Opa protein, which may be homologous to the 28.1 kDa protein identified by SELDI. However, this protein was not listed as a putative protein cross-reactive with *N. lactamica* antisera in chapter 4. High antibody titres have been observed directed towards class 5 proteins (25-30 kDa) in human convalescent sera (Poolman *et al.*, 1983). However, sequences of the Opa proteins vary among meningococcal strains and expression of the proteins is hypervariable. Therefore the use of these proteins as vaccine candidates is limited (Wiertz *et al.*, 1996).

A protein which may have been expected but was not identified as immunogenic by SELDI or the *N. lactamica* genomic library screen is the class 4 protein, RmpM. The function of RmpM is unknown, but the gene is conserved between serogroup A and serogroup B meningococci, with 99.2% identity (Troncoso *et al.*, 2001) and this protein is present in commensal Neisseriae. Antibodies directed towards RmpM, purified from human sera from individuals vaccinated with the Norwegian OMV vaccine reacted strongly with the purified protein but weakly with whole bacterial cells (Rosenqvist *et al.*, 1999). This suggests that antibodies raised towards the vaccine RmpM component are not directed towards surface-exposed regions of RmpM. Therefore any protection elicited by the OMV vaccine may not be due to antibodies directed towards RmpM.

6.2. *Neisseria lactamica* as a Vaccine against Meningococcal Disease.

N. lactamica is involved in natural immunity to meningococcal disease. Gold *et al.*, (1978) examined the correlation between acquisition of *N. lactamica* and the development of cross-reacting antibodies directed towards *N. meningitidis*. Cross-reactive and bactericidal antibodies developed in children who were asymptomatic carriers of *N. lactamica*. Sixty-six percent of children showed an increase in cross-

reactive anitbody titres and 40% showed an increase in bactericidal antibody titres towards meningococci of serogroups A, B and C. They suggested that due to the bactericidal activity of carrier sera some of the cross-reacting antibodies might be involved in protection. However, the results obtained in chapter 3 of this study showed that *N. lactamica* OMPs do not lead to the formation of bactericidal antibodies in mice even though protection was observed. It may be that formation of bactericidal antibodies as a correlate of protection is only reliable for human disease. Therefore the efficacy of *N. lactamica* as a vaccine needs to be assessed in man. It has been shown in this study that as well as protecting mice against lethal meningococcal challenge, antibodies raised against *N. lactamica* are cross-reactive with a broad range of meningococcal isolates. Although many authors have discussed the involvement of *N. lactamica* in protecting naturally against meningococcal disease and the cross-reactivity of antigens between *N. lactamica* and *N. meningitidis*, none have assessed the protective efficacy of *N. lactamica*-based vaccines in any form (Gold *et al.*, 1978, Olsen *et al.*, 1991, Adrade *et al.*, 1986, Kim *et al.*, 1989, Troncoso *et al.*, 2000, Cartwright *et al.*, 1987). The results obtained in this study suggest that *N. lactamica* may provide a suitable vaccine against serogroup B meningococcal disease which is effective independent of serotype and serosubtype.

A *N. lactamica* OMV vaccine may have greater potential as a vaccine against meningococcal disease than a *N. meningitidis* OMV vaccine due to the lack of PorA. PorA in *N. meningitidis* OMV vaccines is immunodominant, implying that responses to other protein components of the vaccine may be poor. For example, although the porin proteins are the most abundant neisserial outer membrane proteins and are present in equal amounts (Blake *et al.*, 1986), vaccination with *N. meningitidis* OMVs in adult volunteers elicits strong T-cell responses to PorA and responses to PorB are significantly lower. Due to the enormous heterogeneity of PorA amongst *N. meningitidis*, meningococcal OMV vaccines can only really be effective if they contain various PorA representing the circulating serosubtypes. PorA would not comprise part of a *N.*

lactamica OMV vaccine, so this vaccine may induce an immune response which is not directed towards an individual protein, but towards a variety of proteins and would be independent of serosubtype. Another advantage of *N. lactamica* as a vaccine against meningococcal disease is that because *N. lactamica* is a commensal organism, its antigens are not under the same selective pressure as its pathogenic relative, *N. meningitidis*. A vaccine produced from meningococcal proteins may require modification over time with the changing antigens of circulating *N. meningitidis* strains in the same way that flu vaccines are updated. For example, TbpB displays extensive sequence divergence throughout the gene, which are likely to have resulted from selective pressure exerted by the immune system (Legrain *et al.*, 1996). A vaccine may target a subset of polymorphic antigens and the efficacy of vaccination with these is determined by the degree of cross-protection afforded by the vaccine (Gupta *et al.*, 1999).

Ala'Aldeen *et al.*, (2000) studied long-term carriage of *N. meningitidis* among university students and development of meningococcal disease. It was found that a student who had carried *N. lactamica* followed by *N. meningitidis* developed meningococcal disease caused by the homologous meningococcal strain. They suggested that carriage of *N. meningitidis* or *N. lactamica* will not necessarily protect against disease development and that the use of *N. lactamica* or attenuated *N. meningitidis* as vaccines should be given cautious consideration. No data was supplied regarding antibody titres, serum bactericidal activity or the health of the patient. Therefore, it is unknown whether this patient ever mounted an immune response prior to disease. *N. lactamica* carriage is common in the meningitis belt of Africa, but it is unclear why the incidence of serogroup A meningococcal disease is so high and carriers of *N. lactamica* in this area do not develop long lasting immunity (Blakebrough *et al.*, 1982).

There has been a great deal of interest in meningococcal OMPs as vaccine candidates either in OMV vaccines or as individual antigens (Sections1.8.4. and 1.8.5.). Of these, only OMVs are licensed as vaccines and the major OMPs show a great deal of

heterogeneity. This study has shown that a number of *N. lactamica* outer membrane components may be useful vaccine candidates due to their homology with meningococcal OMPs and the cross-reactivity of sera raised against them. Whether useful individually or as constituents of a live or OMV vaccine, it is important to characterise the protective components of a *N. lactamica*-based vaccine. Sanchez *et al.*, (2001) suggest however, that use of an OMP-based vaccine may reduce carriage and colonisation by commensal species including *N. lactamica*. This may harm natural immunity and this is only acceptable if vaccine-induced protection is adequate.

6.3. Future Work and Improvements to this Study.

There are a number of ways in which this project can be furthered and improved. Firstly, as bactericidal activity of mouse and rabbit sera raised against *N. lactamica* does not correlate to protection, some other method of protection must be active. It would be of interest therefore to assess the opsonophagocytic activity of these sera as it could provide a better correlate of protection. Clinical trials in man with *N. lactamica* vaccines would provide the most information regarding the protective efficacy of *N. lactamica*. In addition because, the formation of bactericidal antibodies generated by *N. lactamica* carriage has in the past been observed in man, it would be expected that vaccination with *N. lactamica* would induce bactericidal antibodies.

Use of 2D preparative electrophoresis, rather than 1D preparative electrophoresis used in this study, would have provided better information regarding the protective components of *N. lactamica*. However 2D electrophoresis is best for analysis of small groups of proteins rather than the entire proteome of an organism (Graves and Haystead, 2002), so a combination of the two methods would provide most information. Further separation of the LMW protective *N. lactamica* vaccine by 2D-electrophoresis and immunisation of mice with individual proteins from this group could be used to assess immune responses of mice to these proteins. However, the value of this is likely

to be limited due to proteins not being in their native form. This process would also be extremely time consuming, labour intensive and expensive in terms of the animals used.

Chapter 4 showed the use of SELDI to identify meningococcal proteins cross-reactive with *N. lactamica* antisera. 2D-electrophoresis of meningococcal proteins followed by enzymic digestion and then SELDI would provide more useful information regarding the proteins of *N. meningitidis* that cross-react with sera raised against *N. lactamica* OMPs. This is the most important change that could be made and potentially the most fruitful in terms of results that could be made to this project and is discussed in more detail in section 4.3.1.

A *N. lactamica* genomic library was used as an alternative approach to identify the protective components of *N. lactamica*. A more useful approach may have been to screen the library with a variety of different sera against *N. meningitidis*. *N. lactamica* proteins with epitope homology to meningococcal proteins, against which an immune response had been raised, could have been identified directly. However, from the results obtained in chapter 5, it is obvious that the proteins of interest when expressed in recombinant phage are cross-reactive with a variety of human and animal sera.

It would be useful to know the presence and conservation of the proteins identified by screening the *N. lactamica* genomic library in a variety of meningococcal strains i.e. are the proteins and genes identified present and expressed in different serogroups, serotypes and serosubtypes of *N. meningitidis* and are the sequences conserved? Finally, it would be useful to produce recombinant forms of these proteins in large enough quantities to assess their protective efficacy and immunogenicity in animal models and human clinical trials.

6.4. Concluding Remarks.

N. lactamica OMPs of less than 43 kDa have been shown to provide protection to mice against lethal meningococcal challenge. Sera raised against *N. lactamica* OMPs have been used to identify putative *N. lactamica* proteins involved in protection by SELDI and through screening a genomic expression library. SELDI did not identify individual proteins and the proteins putatively identified using this technique did not overlap with those identified using the genomic library screen. However, proteins identified as potential vaccine antigens by other authors were putative proteins identified by SELDI. Using a *N. lactamica* genomic expression library, 23 proteins were identifed as potential vaccine candidates with homology to meningococcal proteins. The aim of this project, to identify the protective components of *N. lactamica* has been fulfilled; potential vaccine candidates have been identified. Further work to assess the protective efficacy of these proteins is required and would provide additional evidence to show how *N. lactamica* protects against meningococcal disease.

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